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THE
BIOLOGICAL BULLETIN

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STUDIES OF THE MITOTIC FIGURE

I. Chaetopterus: Central Body Structure at Metaphase, First Cleavage, After Picro-Acetic Fixation

HENRY J. FRY

(From Washington Square College, New York University, and the Marine Biological Laboratory, Woods Hole)

I. Purpose of the Study

Several years ago, in studies of central bodies in *Echinarachnius* eggs, the writer reached the conclusion that in the cytasters, spermasters, and first-cleavage figures of this egg the central body is a coagulation product of the area of focalized rays and spindle fibers, having no existence as an individualized body in the living cell (Fry, 1928, etc.).

To ascertain whether this situation exists in other forms, a number of studies have now been made of various cell types, including spermatocytes, oöcytes, recently fertilized eggs, early and late blastomeres and somatic cells, in various organisms, ranging from ceolenterates to vertebrates. The central bodies in these cells exhibit such a wide diversity in behavior that the writer has made a provisional classification of centrioles and centriole-like bodies, which is discussed later (p. 181). Each class is identified by some phase of behavior not shared by the others. In the case of each type of cell investigated the purpose was to find out to which class the centriole belongs. After all of these results have been reported, the interrelations of the different classes will be discussed in a later paper.

The material of the present study, which is the first of the group just mentioned, is the egg of *Chaetopterus pergamentaceus*. Mead (1898) described typical centrioles in this egg which maintain genetic continuity from one cell cycle to the next. Wilson (1930) recently re-examined the original preparations of that work and confirmed Mead's observations. Some years ago the writer attempted to duplicate Mead's findings, using Boveri's picro-acetic reagent. Mead states that of the fixatives he employed this gave the best results. In the majority of the cells the writer studied, however, the astral centers were disrupted. A

group of experiments was therefore carried out, using this same reagent, for the purpose of ascertaining what phase of technique was responsible for Mead's demonstration of centrioles on the one hand, and the writer's inability to repeat the work on the other. Study was confined to metaphase asters of first-cleavage figures, because in general central bodies are most readily demonstrated about the time of metaphase, even if they are not present at earlier or later phases; and the mitotic figure of first cleavage has the advantage of its unusually large size.[1]

II. Methods

Treatment of Living Eggs

Prior to fertilization, the eggs were divided into three lots,[2] and fertilized at five-minute intervals, e.g., 9:55, 10:00, and 10:05. They were later mixed and the average time, e.g., 10:00, was regarded as the time of fertilization. Hence when eggs are fixed for the purpose of securing them at metaphase, which occurs in about 52 minutes at 21° C., there is a "spread" of stages from early prophase to late anaphase, and many eggs are in metaphase even if development has been retarded or accelerated in any one egg-set.

After fertilization the eggs were placed in a 1000 cc. crystallizing dish filled with sea water and kept covered to avoid evaporation and consequent modification of osmotic pressure. The water was changed every ten minutes and the eggs were gently stirred every two or three minutes. This avoids possible effects of overcrowding, keeps the oxygen supply normal, and removes metabolic wastes. The dish was immersed in a water bath and kept at a temperature of 21° ± .5. About five minutes before fixation the eggs were transferred to small Stender dishes. Just prior to fixation the water was poured off from each dish, leaving at the bottom a dense mass of eggs, together with a minimum amount of sea water, which necessarily accompanies the eggs. All egg-sets used showed more than 95 per cent first cleavage.

[1] Appreciation is expressed to Miss Sara J. Reynolds for her aid as research assistant in the work.

[2] The parapodia were cut up in a small amount of sea water, then placed on wet cheesecloth drawn taut over an empty bowl, and water was gently squirted through the mass until all eggs were released. Thereafter the water was changed every five minutes for the first half hour, during which maturation reaches metaphase of the first division. The eggs of two or more females were used in each egg-set, and they were fertilized about half an hour after they were secured. The sperm suspension was prepared by adding one drop of thick seminal fluid to about 10 cc. of sea water. Several drops of this were added to the eggs in 250 cc. of water. The exact dilution of the sperm suspension is unimportant, since polyspermy rarely occurs.

Fixation and Slide-making

The picro-acetic reagent employed in the experiments was made according to Boveri's formula (1887, p. 11): 99 parts of a saturated solution of picric acid which has been diluted with two volumes of water, and one part of glacial acetic acid. In experiments where this formula was modified the details are given in each case. Unless otherwise stated, eggs were run up in the usual manner, sectioned at a thickness of 5 μ, and stained in Heidenhain's hæmatoxylin.

Making Observations

It is well known that the two asters of the first-cleavage figure in *Chaetopterus* eggs differ in size (Fig. 1). In this paper only the larger aster of each figure was studied, in order to exclude possible variations due to differences in astral size.

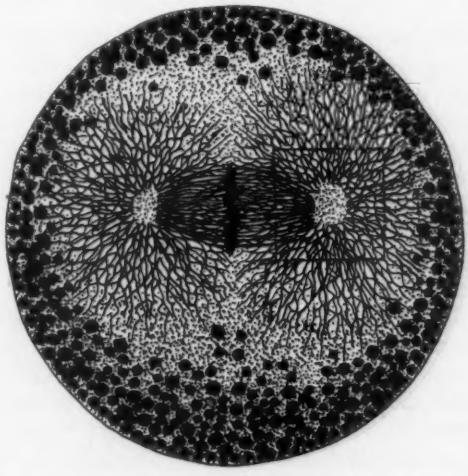


FIG. 1. The metaphase first-cleavage figure in *Chaetopterus* eggs after using Boveri's picro-actetic fixation.

The size difference between the two asters is apparent. The area outlined by the dotted lines is that part of the figure illustrated in the charts. There is here shown that type of structure occurring most frequently after using Boveri's picro-acetic fixation.

Metaphase is here regarded as the time when chromosomes are aligned in a flat plate. Eggs in late prophase, when the chromosomes are in a broad irregular group, and in early anaphase, when they are just beginning to separate, were excluded.

Only the mid-section of the large aster was studied. The sections at the left and right were always examined in order to be sure which was the mid-section. If any section was missing that egg was discarded.

A 4 mm. high dry objective was used in making a list of readings. When several types of central bodies occur on the same slide, as is usually the case, one may stand out more distinctly than the others, especially when such an objective is used. To make sure that a random sample of the egg population of each slide was secured, and to eliminate unconscious selection of any class, the slide was searched systematically by the use of a mechanical stage, and every metaphase figure was listed until the desired number was obtained. These cells were then studied under critical lighting conditions at magnifications of 600 or 900 ×, using an objective having a numerical aperture of 1.4 and a similarly corrected condensor.

Illustrations

In this study, as well as in those to be reported later, it is as important to illustrate the details of ray structure as of central bodies, since the major conclusion of the work is that the structure of the one is closely related to that of the other. Four types of illustration were tried: photographs made with white light, photographs made with ultra-violet light, wash drawings, and ink drawings.

Photographs made with white light show adequately the structure of the central body, but, owing to the nature of the photographic process,[3] they fail completely to show the finer details of ray structure so obvious to the eye.

Through the cooperation of Dr. F. F. Lucas, of the Bell Telephone Laboratories, ultra-violet photographs were made of the asters of *Chaetopterus* and several other species. They will be reported in a later paper, since they will have more significance if discussed after the regular cytological studies have been completed. Such photographs show delicate detail with maximum clarity; but until the technique has been simplified and made less expensive, they cannot have wide-spread use in cytological studies. Hence they are not employed in this group of papers as the regular mode of illustration.

[3] If, for example, a photograph is made of a series of alternating dark grey and light grey lines which have a relative intensity of 10 to 1, the relative intensity of the images of those lines on the negative is 2 to 1. This phenomenon has no practical consequence when photographing such objects as chromosomes or typical centrioles which are in distinct contrast to the surrounding material. But wherever there are only slight differences in intensity, and where the pattern is a delicate one, as in the case of ray and inter-ray materials, a photograph is capable of showing only a vague and unsatisfactory image of the actual structure.

Drawings of any kind, whether wash or ink, have the disadvantage of possibly showing unconscious over-emphasis of certain points or inadvertent omission of others; but they can show delicate details not reproduceable by white light photography, and they have the distinct advantage of showing what the eye sees at various levels of the preparation. In most cytological papers drawings of both astral rays and spindle fibers are frankly schematized, since the exact and literal delineation of the complicated ray pattern presents practically insurmountable obstacles—obstacles not associated with illustrations of most cell components. In many cases, e.g., Mead's wash drawings of *Chaetopterus* asters (1898), the metaphase rays are shown as straight, whereas they are actually more or less undulating. If ink drawings are used, as is often done, where rays are usually shown as black lines and inter-ray materials are not shown at all, the illustration has a degree of contrast much greater than that in the preparation. Furthermore, completely homogeneous areas, such as some large centrosomes, can be shown only by closely-placed dots, which give a granular effect unlike that of the original structure.

Wash drawings are unquestionably more pleasing aesthetically than ink drawings, for the use of several tones of grey and black results in an illustration having a general appearance more like that of the original preparation than is possible when only black is used. But both types can be either accurate or inaccurate in delineation of structure; and when such complex detail as the exact configuration of astral rays is illustrated both wash and ink drawings are inevitably more or less schematized. To show differences in the coarseness and the shape of rays in various astral types, ink drawings are capable of giving as adequate a picture of structural variations as are those made with the wash technique.

Ink drawings have been selected as the mode of illustration in this group of papers, because they can be embodied in charts, reproduceable with zinc plates, which are impracticable when wash drawings are used. Thus variations in astral structure can be related in a graph-like manner to the modifications of various experimental conditions, and there is apparent at a glance the relative frequency of each type under each condition.

Only the central region of the aster is shown in each drawing—an area indicated by the dotted lines in Fig. 1, page 151. If entire cells were shown at the magnification used, 1000 ×, they would occupy too much space to be included in charts.

The drawing of each of the twelve astral types occurring in this study was made from a specific cell, chosen because it was typical of

the class. Each drawing, therefore, not only delineates a single "best" cell, but it also represents the type.

Terminology

Following Wilson's usage, (1928, pp. 30 and 672–675) the term *centriole* is used to indicate a minute darkly-stained granule-like body; the term *centrosome* refers to a larger, more variable structure that often surrounds the centriole or may exist by itself. A *centrosphere* is a large vacuolar area at the astral center. The term *central body* is a general one; it may apply to any or all of these structures, and hence includes all configurations from a minute granule to a large empty area.

Mead uses these terms with different meanings: what is here termed a *centriole* he calls a *centrosome,* and what is here termed a *centrosome* he calls a *centrosphere.*

III. Experiments

Group A. Technique Standard: Boveri's Picro-Acetic Fixation and Regular Slide-Making Procedure

Experiment 1. The Relation Between Central Body Structure and Ray Structure

In this experiment the 243 eggs studied were selected at random from slides of various egg-sets which had been fixed, sectioned, and stained under supposedly similar optimum conditions. Preliminary examination having shown several types of central bodies present, an attempt was made to ascertain whether or not these variations in central body structure could be related to other structural modifications of the mitotic figure. To that end the large metaphase aster in each cell was analyzed with reference to the following points:

I. The central body
  A. The centrosome
    1. Physical structure (empty, containing more or less scattered materials, or evenly homogeneous)
    2. Size and shape
    3. Degree of demarcation from ray area (demarked distinctly, doubtfully, or not at all)
    4. Stain in contrast to that of ray area (lighter, similar, or darker)
  B. The centriole
    1. Number
    2. Location in the centrosome
    3. Size
    4. Shape (regular or irregular)
    5. Contour (smooth or rough)
    6. Stain in contrast to that of the centrosome

C. Granules, other than centrioles, occurring near the astral center
   1. Number
   2. Size
   3. Location
   4. Similarity to cytoplasmic granules

II. The rays [4]
   A. Coarseness (very coarse, medium coarse, delicate, or vague)
   B. Shape (rippled or serpentine, undulating or almost straight)
   C. Occurrence of small vacuole-like areas among the rays

III. The spindle
   A. Size
   B. Shape of tip (pointed, rounded, or intermediate)
   C. Structure of fibers in contrast to that of astral rays

The various types of centers occurring after Boveri's picro-acetic fixation constitute an unbroken series: the central area may be completely empty; it may contain either small amounts of scattered material, sometimes arranged like the walls of vacuoles, or more abundant material, distributed regularly or irregularly; finally, it may be an evenly-filled, homogeneous region. These centers are about $5 \times 6\,\mu$ in size, although there is much variation here.[5] Whether such a series is illustrated by few or many drawings is purely arbitrary. In Chart I three classes are shown: "empty," "scattered," which contain more or less irregularly distributed material, and "even," or homogeneously filled centers, which merge gradually with the ray area and are stained like it.

The various ray configurations also constitute a continuous series: at one extreme they are "rippled" or serpentine; at the other they are "undulating" or almost straight; the intermediate type can best be described as "slightly rippled." The rippled rays have many minute clear spaces, like tiny vacuoles, among their deep curves; the undulating ones have few or none.

Thus when *Chaetopterus* eggs in metaphase are fixed with Boveri's reagent there is a definite relation between central body structure and

[4] It is difficult to select concise terms that describe clearly the differences in the coarseness of rays and in their shape. For example, the use of "very coarse" in contrast to "medium coarse" is somewhat clumsy, but it is necessary to express two degrees of coarseness as contrasted with the condition described by the term "delicate." The terms "rippled" and "undulating" were selected because the former suggests the idea of short sharp curves, in contrast to the long gentle curves suggested by the latter.

[5] Even among cells in metaphase which are alike in the general structure of rays and central bodies there is considerable variation in the size of the center. It is practically impossible to secure accurate measurements in most cases, because the central body is demarked from the ray area either vaguely or not at all. In each type, therefore, the cell selected for illustration has a central body of about the average size for its class, based on attempted measurements of about twenty figures of that type. There is also much variation in the shape of the centers: some are round, others quite elongate, others intermediate. The cells selected for illustration as representative for each type have central bodies that are intermediate in shape, being but slightly elongate.

ray structure: all asters with rippled rays have disrupted centers that are either empty or scattered; all those with undulating rays have even centers that are homogeneously filled. There are no exceptions to this relationship. Asters with slightly rippled rays, which are intermediate between these two classes, show all types of centers.

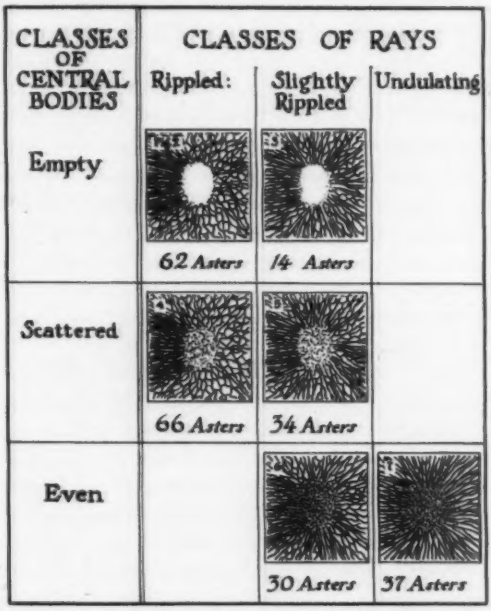


CHART 1. THE RELATION BETWEEN RAY STRUCTURE AND CENTRAL BODY STRUCTURE UNDER OPTIMUM CONDITIONS

Central bodies were studied in metaphase first-cleavage figures in *Chaetopterus* eggs, after using Boveri's picro-acetic fixation, and the standard slide-making procedure. *Result:* Central body structure is related to ray structure: centers are "disrupted", *i.e.*, empty or containing scattered material, if rays are rippled in shape; centers are "even", *i.e.*, homogeneously filled and stained like the ray area, if rays are undulating. Centrioles are not demonstrated.

The slightly rippled rays are obviously intermediate between rippled and undulating rays. Since typically rippled rays are always associated with disrupted centers (empty or scattered) and typically undulating ones with evenly filled centers, the members of this intermediate, or slightly rippled group will hereafter be included with either the rippled or the undulating class, depending upon which group the aster most resembles. Thus the four classes of asters illustrated in Chart 1, in which the centers are empty or scattered and rays either

rippled or slightly rippled (Figs. 2–5) will hereafter be regarded as but minor variations of a single major type, and represented in later charts by an illustration showing a disrupted (scattered) center and rippled rays (Fig. 4). Similarly, the two classes with even centers and rays either slightly rippled or undulating (Figs. 6 and 7) will hereafter be regarded as variations of another major type, represented in later charts by an illustration showing an even center and undulating rays (Fig. 7). These two major types of asters are but two of twelve that occur in this investigation, all of them illustrated in Chart 7 (p. 177). The type with disrupted centers is designated as 1*B* and that with even centers as 2*B*. The basis of classification is discussed on page 176.

Very rarely, *i.e.*, in 16 of the 243 asters studied, one or more granules, like the smaller ones present in the cytoplasm, occur in the even type of center. But since they vary in size, location, and staining capacity, they could not be interpreted as centrioles. These random granules are not the structures Mead illustrated; the kind of central body he described was not produced in this experiment, in which Boveri's picro-acetic reagent was used in the usual way.

Experiment 2. The Effects of Uncontrolled Factors in the Handling of Different Egg-sets Under Optimum Conditions

The purpose of this experiment was to learn whether the percentages of disrupted and even centers (Types 1*B* and 2*B*) occurring in one set of eggs handled under optimum conditions are the same as those occurring in other egg-sets run under supposedly similar conditions but on different days. Table I shows counts of these two types of centers in five different sets, each sample including from 33 to 67 eggs. These data are arranged in the order of increasing percentages of the disrupted type.

When Sets 1 and 2, 2 and 3, 3 and 4, and 4 and 5 are compared, there are only minor differences in the percentages of the two central body types present, and these could be explained by the relatively large errors always involved in reporting small samples. But when Sets 1 and 3, 2 and 4, and 3 and 5 are compared, the differences are large enough to suggest some cause beyond error of sampling. And when Sets 1 and 4, 1 and 5, and 2 and 5 are contrasted it is obvious that the discrepancies are too great to be explained by errors of sampling alone; they must be due either to differences between the living eggs of the various sets prior to fixation, or to uncontrolled modifications of technique. When these relations are analyzed statistically, by determining the value of *P* according to Pierson's method (1924, p. lxx) the ex-

istence of factors which cause differences, other than errors due to small samples, is convincingly demonstrated.[6] The approximate values of P for this material are shown in Table I.

Three problems present themselves: (1) to secure more data concerning the relation between central body structure and ray structure; (2) to explain the cause of the difference in the relative numbers of disrupted and even centers present in different sets after picro-acetic fixation under optimum conditions; and (3) to ascertain what variation of the picro-acetic technique Mead used to demonstrate typical centrioles. The experiments which follow were planned in an attempt to secure information on these points.

Group B. Technique Modified: Slide-Making Procedure Varied; Boveri's Picro-Acetic Fixation

Experiment 3. The Effects of Varying the Depth of Stain (Heidenhain's Hæmatoxylin)

It is conceivable that a center containing considerable irregularly scattered material might, if lightly stained, be listed as belonging to the disrupted (scattered) type, while the same center, if darkly stained, might appear to be evenly filled and would then be counted as an even type. In that event, the differences in the relative numbers of disrupted and even centers in the five sets of the previous experiment might be due to differences in depth of stain.

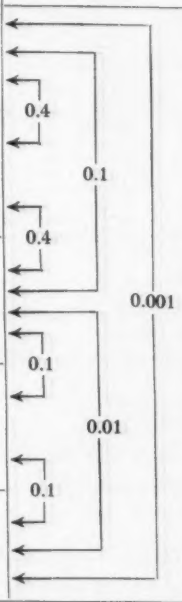
In that experiment the eggs were stained a deep blue color with Heidenhain's hæmatoxylin. Table II repeats the data of that experiment at the left, and also shows, at the right, the numbers of disrupted and even centers which occurred when eggs of the same five sets were stained a very pale blue. In each set all experimental conditions were identical, in so far as they could be controlled, except the depth of stain.

[6] Pierson's method determines whether a given sample of objects (the 67 eggs of Set 1) having a given number of one class (40 disrupted centers) and a given number of a second class (27 even centers) does or does not belong to the same population as another sample (the 55 eggs of Set 2) with different numbers of the same classes (36 disrupted centers and 19 even centers). These relations are expressed in terms of P. Thus the value of P for Sets 1 and 2, as well as for Sets 2 and 3, is 0.4, meaning that there are 4 chances out of 10 that each pair belongs to the same population, and indicating that the differences between them are not significant. At the other extreme, however, the value of P for Sets 1 and 4 is 0.02, and for Sets 1 and 5 it is 0.001, showing that the chances are, respectively, one out of 50 and one out of 1000, that each pair belongs to the same population. The differences between these sets are therefore significant.

TABLE I

The effects of uncontrolled factors in the handling of different egg-sets under optimum conditions. Central bodies were studied in metaphase first-cleavage figures in *Chaetopterus* eggs, after using Boveri's picro-acetic fixation, and the standard slide-making procedure. *Result:* Some factor or factors are involved which modify significantly the numbers of the two central body types in the different egg-sets.

Egg-Set Symbol	Number of Cells Studied	Distribution of Astral Types in Each Set		P
		1 B (Disrupted)	2 B (Even)	
1	67	40 60%	27 40%	
2	55	36 65%	19 35%	
3	48	34 71%	14 29%	
4	33	26 79%	7 21%	
5	39	35 90%	4 10%	



It is apparent, both by inspection and by the statistical determination of the value of *P*, that the depth of stain with Heidenhain's hæmatoxylin does not modify significantly the percentages of the two central body types occurring in any one egg-set after Boveri's picro-acetic fixation. This is especially obvious when comparing the totals.[7]

[7] This fact does not indicate that various depths of stain would be equally ineffective in modifying the appearance of other central body types, to be described later.

TABLE II

The effects of varying the depth of stain (Heidenhain's hæmatoxylin). Central bodies were studied in metaphase first-cleavage figures in *Chaetopterus* eggs, after using Boveri's picro-acetic fixation. The slide-making procedure was standard except that egg-sets reported at the left in the table were stained darkly and those at the right were stained lightly. *Result:* Differences in depth of stain do not significantly modify the numbers of the two central body types occurring in the different egg-sets.

EGG-SET SYMBOL	EGGS STAINED DARKLY			P	EGGS STAINED LIGHTLY		
	Number of Cells Studied	Distribution of Astral Types in Each Set			Number of Cells Studied	Distribution of Astral Types in Each Set	
		1 B (Disrupted)	2 B (Even)			1 B (Disrupted)	2 B (Even)
1	67	40 60%	27 40%	←0.8→	36	21 58%	15 42%
2	55	36 65%	19 35%	←0.9→	42	27 64%	15 36%
3	48	34 71%	14 29%	←0.6→	44	33 75%	11 25%
4	33	26 79%	7 21%	←0.7→	48	39 81%	9 19%
5	39	35 90%	4 10%	←0.1→	73	61 84%	12 16%
TOTALS	242	171 71%	71 29%		243	181 74%	62 26%

Experiment 4. The Effects of Other Modifications of the Slide-making Procedure

Wide variations as to the speed with which the eggs are passed through the alcohols and xylol, the temperature at which they are embedded, and the thickness at which the ribbon is sectioned, do not change the appearance of the central bodies when Boveri's picro-acetic reagent and Heidenhain's hæmatoxylin are used.

The effects of modifying one other phase of the slide-making procedure were also studied—accidental overheating of the ribbon when it is warmed to bring about its expansion. Different samples of ribbon were variously treated during this operation: in one the ribbon was expanded in the usual manner, remaining opaque without melting throughout the operation; another was so overheated that the ribbon melted

and became transparent for a moment; in a third the same situation was produced but maintained for a longer time; finally, one slide was so extremely overheated that the ribbon not only melted but bubbled and gave off vapors. In the latter case a number of the eggs have a peculiar glassy appearance in the region of the spindle, the inner zone of the astral rays, and the central body area (Fig. 8). This type, produced by faculty technique, is readily recognized and could not be confused with the types occurring under usual conditions.

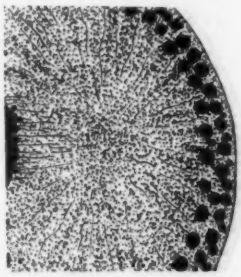


FIG. 8. The glassy type of astral center caused by overheating the ribbon when expanding it.

Central bodies were studied in metaphase first-cleavage figures in *Chaetopterus* eggs, after using Boveri's picro-acetic fixation. The slide-making procedure was standard, except that the ribbon was overheated when expanding it. *Result:* The central body area, the inner part of the ray region, and the spindle have a glassy appearance.

It can therefore be concluded that when *Chaetopterus* eggs are fixed with Boveri's reagent, the inevitable slight variations in the slide-making procedure do not modify central body structure. While this conclusion applies only to the disrupted and even types, it is probable that it also holds true for the other types to be described.

GROUP C. TECHNIQUE MODIFIED: PICRO-ACETIC FIXATION VARIED; REGULAR SLIDE-MAKING PROCEDURE

Experiment 5. The Effects of Varying Simultaneously the Amounts of Picric and Acetic Acids

When eggs are added to the fixative in a vial, even the densest egg suspension includes some sea water, which slightly dilutes the reagent. The present experiment deals with the effects of a series of dilutions, beginning with one part of distilled water and 99 parts of reagent, and ending with 99 parts of water and one part of reagent. To test-tubes

containing 25 cc. of each dilution were added 0.5 cc. of eggs. The amount of sea water included with the eggs was so small, compared with the 25 cc. of diluted fixative, that the effects of any further dilution could safely be ignored. The eggs fixed at 99 per cent, 90 per cent, 75 per cent, 50 per cent, 10 per cent and 1 per cent strength were from one egg-set; those fixed at 30 per cent, 20 per cent, 15 per cent, and 5 per cent were from another egg-set.

Chart 2 shows the percentages of the central body types occurring after fixation with each dilution. When the reagent is from 99 per cent to 50 per cent strength the disrupted and even types previously described (*1B* and *2B*) are the only ones present, their numbers varying at the different dilutions. At 30 per cent strength two new types appear, one having a vaguely delimited centrosome stained darker than the ray area, but without a centriole (*4A*), and one with a similar centrosome but containing one or two centrioles (*5A*). Between 30 per cent and 5 per cent strength these are the major classes. At 5 per cent strength a new type of aster makes its appearance, similar to the one with an even center so frequently described except that it has very delicate rays (*2C*). At 1 per cent strength the asters are very small and vague and their centers are undifferentiated from the ray area. Cytasters are also present,[8] and the chromosomes are abnormal.

The relation between ray structure and central body structure is again obvious, and additional evidence on this point is supplied by the new types. (1) If rays are fixed vaguely, the central area of the aster is entirely undifferentiated from the peripheral part (*2D*). (2) If rays are distinct and rippled the center is disrupted (*1B*). (3) If rays are distinct but undulating, the astral center is filled, its structure varying with the coarseness of the rays, as follows: When rays are either delicate or medium coarse, the center is a homogeneous centrosome, about $5 \times 6\,\mu$ in size, not delimited from the ray area, and stained like it (*2B* and *2C*). When rays are very coarse there are two classes of centers: (*a*) a centrosome, about $4 \times 5\,\mu$ in size, vaguely delimited from the ray area, more darkly stained, and containing no centriole (*4A*); and (*b*) a similar centrosome but with a centriole, either single or double (*5A*). The possible significance of these facts will be discussed after the data of all the experiments have been presented.

The writer reported these results of diluting the reagent to Pro-

[8] As is well known, cytasters are usually produced by methods which bring about artificial parthenogenesis in unfertilized eggs. Occasionally, however, they have been described in normally fertilized eggs. It is possible that some of these were produced by the use of a dilute reagent, as an accidental result of the mode of fixation.

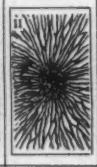
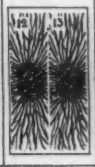
EXPERIMENTAL DATA			DISTRIBUTION OF ASTRAL TYPES AT EACH DILUTION					
			Type 1B	2B	4A	5A	2C	2D
								
DILUTION FORMULÆ — Boveri's Picro-acetic Reagent (c.c.)	DILUTION FORMULÆ — Distilled Water (c.c.)	NUMBER OF CELLS STUDIED						
99	1	34	32 94%	2 6%				
90	10	34	29 85%	5 15%				
75	25	46	31 67%	15 33%				
50	50	82	80 98%	2 2%				
30	70	39	5 13%	5 13%	12 31%	17 43%		
20	80	52	6 12%	6 12%	19 36%	21 40%		
15	85	41	1 2%	4 10%	10 25%	25 61%	1 2%	
10	90	47			6 13%	41 87%		
5	95	47			19 40%	17 36%	11 24%	
1	99	40						40 100%

CHART 2. THE EFFECTS OF VARYING SIMULTANEOUSLY THE AMOUNTS OF PICRIC AND ACETIC ACIDS

Central bodies were studied in metaphase first-cleavage figures in *Chaetopterus* eggs, after fixation with Boveri's reagent diluted to various degrees with distilled water. The slide-making procedure was standard. *Results:* (1) Central body structure is related to ray structure, depending upon the coarseness and shape of the latter. (2) Mead (1898) demonstrated centrioles by diluting the reagent. (3) Uncontrolled dilution effects, resulting from the manner in which eggs are added to vials under usual conditions (Exp. 2) are probably responsible for the variations in numbers of the central body types in different egg-sets.

fessor Mead, suggesting that possibly a comparable dilution might have been responsible for the centriole phenomena he found, and hence for the writer's failure to repeat the work with a full-strength reagent. Professor Mead replied that dilution did occur in his experiments, that the results in different preparations were not uniform, and that in many eggs the centers were disrupted. His failure to mention this dilution in his paper is undoubtedly due to the fact that he attached no significance to it—a situation which occurs almost universally in the case of many supposedly minor variations in different steps of the cytological technique. It is worth noting, however, that diluting the reagent is the only modification of the many employed in this investigation which demonstrates centrioles.

Where Mead used sea water to dilute the fixative, distilled water was used in the present work. It is probable that both kinds of dilutions produce similar results, for centrioles can be demonstrated after dilution with either. It is possible, however, that certain differences may exist.

Varied dilution of the reagent explains the occurrence of disrupted and even types of central bodies side by side on the same slide, as well as the variations in their relative numbers in various egg-sets supposedly fixed and run up in the optimum manner (Table I, p. 159). Uncontrolled dilution effects, differing from vial to vial and modifying the central area in asters, are brought about by slight differences in the manner in which eggs happen to be added to the reagent in the vial. If eggs are squirted quickly from the pipette into the vial containing the reagent the mixing is practically instantaneous, but the eggs are handled rather violently. Furthermore, the dish containing the living eggs may become contaminated if the tip of the pipette, which must be held close to the vial when the eggs are added, is splashed with minute droplets of the reagent. For these reasons, eggs are usually allowed to drop gently out of the pipette. Under such conditions the eggs and any sea water accompanying them are more or less segregated near the top of the vial for the first few seconds, the reagent mixing with them from below. It will be shown later (Experiment 9) that fixation occurs within one second; hence the eggs added to the vial are exposed during the first second to various dilutions of the fixative, depending upon whether they happen to be at the top or at the bottom of the mixture of eggs and sea water just added to the reagent.

The data of Chart 2 show that with few exceptions fixation with a full-strength reagent produces disrupted centers (*1B*), and that progressively greater dilutions of the reagent result in filled centers (*2B*, *4A*, and *5A*). In the preceding experiment (Table I) such effects were in-

advertently produced by the manner in which the eggs of the five egg-sets were added to their vials. In Set 1 a considerable percentage of the eggs undoubtedly received a somewhat dilute fixation, since 40 per cent of them have even centers; but those of Set 5 were added in such a manner that only 10 per cent of them were so fixed.

The effect of varied dilutions also explains why, under usual conditions of fixation, about 70 per cent of the centers are disrupted, the remaining ones being even (Table II). Since *Chaetopterus* eggs frequently stick to the bottom of the vial, the writer has heretofore used a narrow vial (9 mm. wide, inside diameter, and 4.5 cm. high), because in it the eggs pile up on top of each other, and few of them touch the bottom. Prior to fixation these vials contained from 4 to 4.5 cc. of reagent; at the time of fixation from 0.5 to 1.0 cc. of eggs were added. The strength of the reagent thus varied from 80 per cent to 90 per cent, the average being about 85 per cent. In the five sets fixed under these usual conditions, 71 per cent of the eggs have disrupted centers and 29 per cent even ones—a result which is comparable to that of the set fixed at 75 per cent strength in the dilution experiment (Chart 2), where the percentages are 67 and 33.

In this dilution experiment the major differences in types and numbers of central bodies occurring at any given strength of the reagent are without doubt primarily the result of the varied dilutions. Within each set, however, the numbers and proportions of central body types were probably also modified by the exact way in which the eggs were added to the reagent. Until the results of this experiment were known the writer had not realized what radical effects can be produced by dilution. Had the eggs been expelled suddenly from the pipette into the fixative instead of being dropped in gently, not only in the present experiment but in the others as well, it is probable that in many cases the numbers of types would have been reduced and their relative percentages somewhat altered.[9]

The fact that dilution of Boveri's picro-acetic reagent causes such marked differences in the structure of rays and central bodies does not mean that this would be true of all cells fixed with any dilute reagent. However, it is probable that the cells of various species are susceptible to dilution effects in various fixatives. For example, although *Echinarachnius* eggs ordinarily show large pleuricorpuscular central bodies

[9] The existence of such an uncontrolled cause of variation in central body types makes it useless to study large egg samples in each group for the sake of reducing the error inherent in small samples. For this reason the number of eggs studied at each variation is seldom more than 50, the average being 36. But although uncontrolled dilution effects may modify the percentages of any type occurring in the eggs of any vial, they do not alter the relation between central body structure and ray structure, which always holds true, without exception.

when fixed with various full strength reagents, including Bouin's, this fluid diluted with 90 parts water produces a minute centriole surrounded by a homogeneous centrosome. The second paper on *Chaetopterus* eggs will demonstrate that the effects of diluting certain chemically diverse reagents are similar to those of picro-acetic dilutions, although the details differ in each case.

Whether dilution effects have played an unsuspected rôle in central body studies of other species remains to be ascertained by future investigations. It may be that in many eggs extensive dilution of the reagent does not modify astral structure. One egg, however, that may be affected in this way is that of *Ascaris* (Fogg, 1931 and many earlier papers); its chorion is so impermeable that it can live in certain strong reagents for hours. When the one usually used, a chloroform-alcohol-acetic mixture, does finally penetrate, it is quite possible that only a very dilute amount of the reagent as a whole, or of one of its components, actually brings about the fixation. This can be determined only if a technique can be developed whereby *Ascaris* eggs can be subjected to instantaneous fixation with full-strength reagents.

Another egg where dilution effects probably occur is that of *Drosophila* (Wilson and Huettner, 1931). It too is impermeable to the reagent. Therefore it is pricked at one end to permit the fixative to enter, and thirty to sixty seconds are required for it to traverse the egg. This may explain the variations found in different mitotic figures in the same stage. In different eggs the variations may be determined by the size of the puncture, which governs the rate at which the reagent enters; but similar effects also occur in different parts of the same egg, probably depending on various dilution effects at different distances from the point of puncture. Here again it would be interesting to compare the results produced by a technique permitting instantaneous, full strength fixation, with those of the present method.

The fact that a certain configuration is produced by a given dilution of a reagent but is not shown when that fixative is used full strength, argues neither for nor against the validity of that coagulation product. Such differences, produced by different dilutions of the same fixative, may have neither more nor less significance than differences produced by chemically diverse reagents. Nevertheless, if certain configurations are produced only by a diluted reagent, that fact must be kept in mind.

The manner in which significant variations are produced in *Chaetopterus* eggs by diluting the reagent suggests that certain precautions should be followed generally in fixing eggs. The amount of reagent should be large enough to prevent any appreciable dilution by the addition of any fluid accompanying the eggs. Furthermore, the eggs should

be added in such a way as to effect instantaneous mixing, in order to prevent inadvertent dilution effects during the first second, when the eggs are fixed.

Experiment 6. The Effects of Varying in Turn the Amounts of Picric and Acetic Acids

In the preceding experiment the amounts of both acids in the reagent were progressively and simultaneously reduced. The present experiment was carried out in order to study the effect on central body structure when each acid in turn is kept constant while the other is varied.

Four egg-sets, run on different days, were used in this experiment. Each illustration in Chart 3 shows the astral configuration resulting from fixation with a different combination of picric and acetic acids. The figures, grouped in four series, are arranged in the order of percentage by weight of both acids present in each case. In series A, shown by the top horizontal row of figures, the picric acid, used as a saturated solution, was kept constant at about 1.2 per cent, and the acetic varied from 0.007 per cent to 20 per cent, the picric here being three times as strong as in Boveri's picro-acetic formula. In Series B, shown by the second horizontal row of figures, the saturated picric acid solution was diluted with two volumes of water (a mixture designated on the chart as "⅓ picric"). This is the same concentration used in Boveri's reagent, which is 0.4 per cent by weight. In Series C, illustrated by the vertical line of figures near the center of the chart, the acetic acid was kept constant at 1 per cent, its concentration in Boveri's formula, while the picric was varied from 0.01 per cent to 1.2 per cent.[10] In Series D, shown by the oblique row of figures in the lower left-hand corner of the chart, both acids were varied, beginning with picric at 0.4 per cent when acetic is at 1.0 per cent, and ending with picric at 0.003 per cent and acetic at 0.01 per cent. This last series, which was reported in the previous experiment, Chart 2, is included here for purposes of comparison with the other three series. Chart 3 also shows the central body type present after fixation with a 1 per cent solution of acetic acid containing no picric acid (Fig. 43), as well as that occurring after fixation with "⅓ picric," containing no acetic acid (Fig. 44).

Table III shows the various formulæ employed. In calculating the

[10] In Chart 3, the picric acid of Series A and B is spoken of as being constant, but this is only relatively true. In Series A, for example, while the acetic acid was varied from 0.007 per cent to 20 per cent, the picric was varied only from 1.2 per cent to 1.1 per cent and thus was relatively constant. The same situation applies to Series B. In Series C the acetic acid actually was kept constant, since 1 cc. of it was added to 99 cc. of different dilutions of picric acid solution.

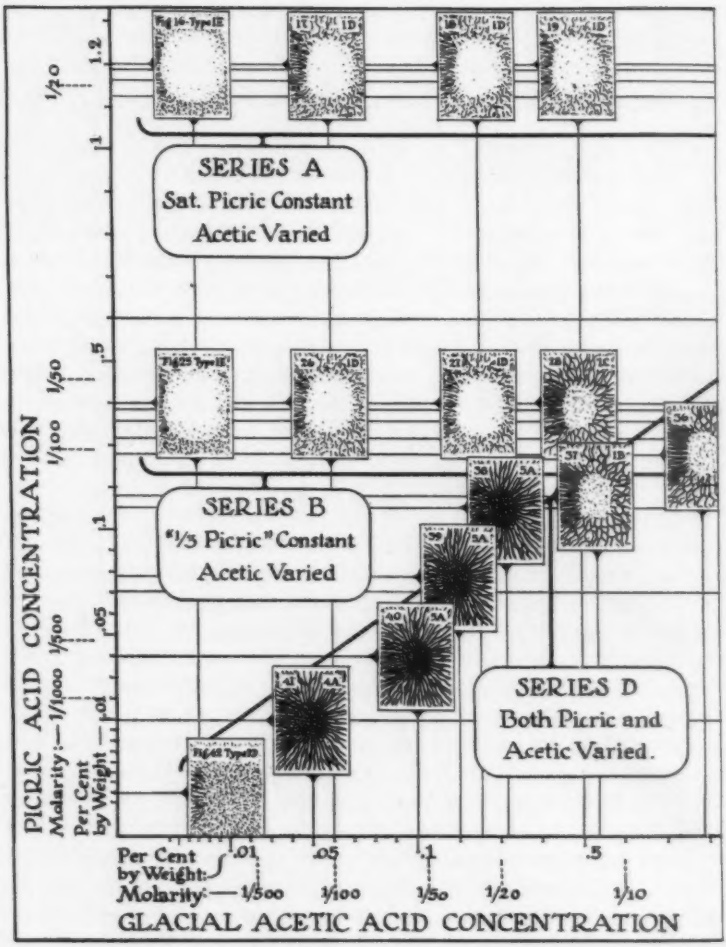
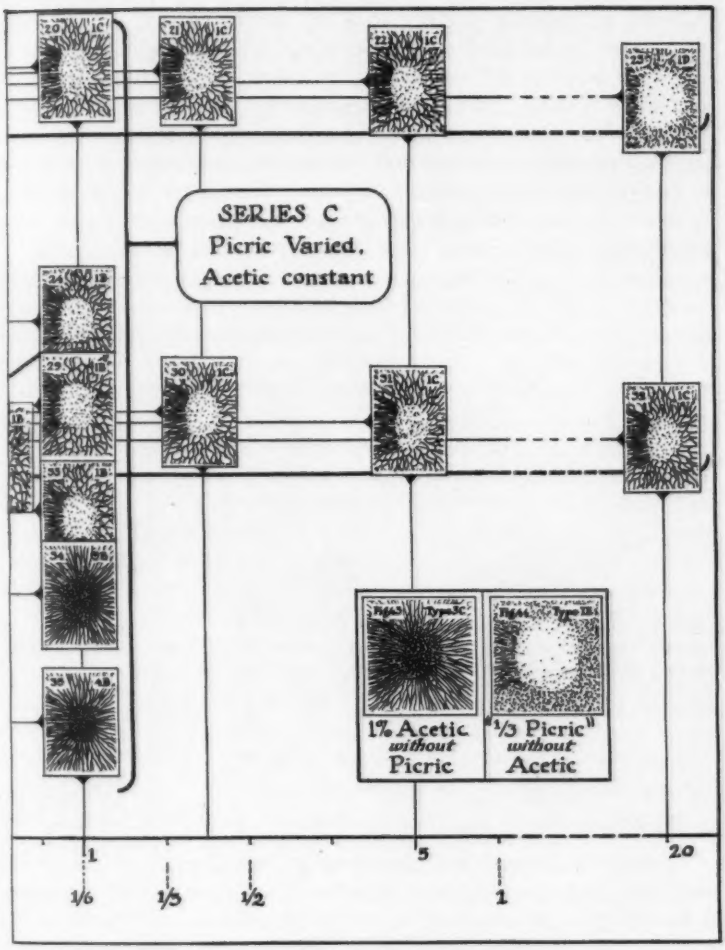


CHART 3. THE EFFECTS OF VARYING IN TURN

Central bodies were studied in metaphase first-cleavage figures in *Chaetopterus* eggs, after fixation with various modifications of the picro-acetic reagent. In some the amount of picric acid was kept constant while the acetic acid was varied (Sets A and B); in others the reverse was done (Set C). The data of the preceding



THE AMOUNTS OF PICRIC AND ACETIC ACIDS

dilution experiment are included for comparison (Set D). The slide-making procedure was standard. *Result:* Only under a very narrow set of conditions are centrioles demonstrated and rays fixed in an undulating and very coarse configuration.

percentage by weight in each formula, 1 cc. of glacial acetic acid was regarded as weighing 1 gram, since its specific gravity is 1.05. The effect upon the solubility of the picric acid (1.22 grams are soluble in 100 cc. of water at 20° C.) caused by changes in room temperature, as well as the possible effect of the presence of acetic acid in the mixture, was ignored. Extreme accuracy in making up the formulæ is meaningless, since slight unknown dilutions were brought about in each case by the addition of small amounts of sea water with the eggs at the time of fixation. The molarities of the two reagents are also shown; they too are only approximately correct.

TABLE III

Formulæ of the picro-acetic reagents used in the experiments reported in Charts 2 and 3

Series A SATURATED PICRIC CONSTANT: ACETIC VARIED			Series B "1/3" PICRIC CONSTANT: ACETIC VARIED			Series C PICRIC VARIED 1% ACETIC CONSTANT					Series D BOTH PICRIC AND ACETIC VARIED		
						Formulæ of Picric Solutions							
Sat. Picric	Acetic	Fig.	Sat. Picric Diluted with 2 Parts Water	Acetic	Fig.	Pts. Sat. Picric	Pts. Water	Picric Sols.	Acetic	Fig.	Picro-Acetic Reagent	Water	Fig.
cc.	*cc.*		*cc.*	*cc.*				*cc.*	*cc.*		*cc.*	*cc.*	
99.99	.007	16	99.99	.007	25	100	0	99	1	20	99	1	29
99.94	.06	17	99.94	.06	26	50	50	99	1	24	75	25	36
99.75	.25	18	99.75	.25	27	33	67	99	1	29	50	50	37
99.5	.5	19	99.5	.5	28	12	88	99	1	33	30	70	38
99.	1.	20	99.	1.	29	6	94	99	1	34	20	80	39
97.5	2.5	21	97.5	2.5	30	1.5	98.5	99	1	35	10	90	40
95.	5.	22	95.	5.	31						5	95	41
80.	20.	23	80.	20.	32						1	99	42

In order to accommodate the drawings, the abscissa of the chart, which shows the percentage of acetic acid, is drawn to various scales in its different parts; *i.e.*, 1 cc. = ½ inch; 0.1 cc. = ¼ inch; 0.01 cc. = ⅛ inch; 0.001 cc. = 1/16 inch. The ordinate showing the percentages of picric acid has been similarly adjusted.

In Series A, B, and C, where there is only slight variation in structure at each modification of the reagent, the illustration on the chart represents the type which is most abundant, based on a study of about 20 eggs in each case. In Series D, where the variation is considerable, large numbers of eggs were studied, but again only the major class occurring after fixation with each dilution is shown, since all of the types occurring are illustrated in Chart 2.

Six new astral types occur in this experiment: (1) one having a disrupted center and delicate rippled rays (1*C*, Figs. 20–22, 28, and 30–32); (2) one with a dense center, stained more darkly than the ray area but not delimited from it, with medium coarse, undulating rays (3*B*, Fig. 34); (3) a similar aster with delicate rays (3*C*, Fig. 43); (4) one with a slightly demarked center, darker than the ray region, with medium coarse, undulating rays (4*B*, Fig. 35); (5) one with a disrupted center accompanied by vague or doubtful rays (1*D*, Figs. 17–19, 23, 26, and 27); and (6) a similar center accompanied by a ray area that is entirely non-radial (1*E*, Figs. 16, 25, and 44).

The results of this experiment are as follows: (1) A typical centriole and its accompanying very coarse undulating rays can be demonstrated only when the percentage by weight of the picric acid is between about 0.01 per cent and 0.2 per cent, and the acetic is at the same time between about 0.05 per cent and 0.4 per cent. Other types of central bodies and ray configurations also occur within this range. (2) If the concentration of the acetic acid is extended to about 2.0 per cent and the picric is held within the range just mentioned, the centers, which are accompanied by medium coarse, undulating rays, are darker than the ray area and are either undemarked from it, *i.e.*, dense (3*B*, Fig. 34), or slightly demarked from it (4*B*, Fig. 35). (3) If the concentration of the picric acid is extended beyond about 0.2 per cent, and that of the acetic beyond about 2.0 per cent, the centers are disrupted and the rays rippled, being either coarse (1*B*), delicate (1*C*), vague (1D), or absent (1*E*), depending upon the formula used. (4) If both picric and acetic acids are very dilute, the picric being less than about 0.005 per cent and the acetic less than about 0.01 per cent, the aster shows only a vague radial organization (2*D*, Fig. 42).

This experiment establishes the fact that the demonstration of a centriole, which is always accompanied by very coarse undulating rays (5*A*), occurs only when the picric and acetic acids are simultaneously within a certain narrow range of concentration.

Experiment 7. The Effects of Varying the Hydrogen Ion Concentration of Boveri's Reagent

Chart 4 shows the effects of the addition of varying amounts of normal sodium hydroxide solution to Boveri's picro-acetic fixative. The pH reported in each case [11] was modified to a slight but unknown degree by the addition of the small amount of sea water which always accompanies the eggs at the time of fixation. In this experiment, however, the egg samples were kept unusually small, so as to change the

[11] The hydrogen ion concentration was determined electrometrically by Mr. Delafield Dubois, who used an apparatus with glass electrodes.

Amounts of normal sodium hydroxide solution listed in Chart 4 were added to 40 cc. of Boveri's reagent.

pH as little as possible. All eggs used in this experiment were from the same egg-set.

The two common central body types previously described occur after using the reagent at its natural pH, 2.2. When the pH is increased to 3.9 the rays are largely suppressed, but the centers remain the same (1*D* and 2*D*). When the pH is 4.7, 5.3, or 5.7, rays are undulating and delicate, while the centers are either even (2*C*) or dense (3*C*). In all sets except the one in which the pH was unmodified the mitotic figures are very small, the chromosomes poorly fixed, and the cytoplasm, except in the region of the mitotic figure, crowded with large, darkly-staining granules. A definite relation between ray structure and central body structure is again shown.

EXPERIMENTAL DATA			DISTRIBUTION OF ASTRAL TYPES AT EACH pH					
			Type 1B	2B	1D	2D	2C	3C
pH	c.c. of Normal NaOH added to Boveri's Reagent	Number of cells studied						
2.2	0	20	14	6				
3.9	2	19			15	4		
4.7	4	23					17	6
5.3	6	23					7	13
5.7	7	14					14	

CHART 4. THE EFFECTS OF VARYING THE HYDROGEN ION CONCENTRATION OF BOVERI'S REAGENT

Central bodies were studied in metaphase first-cleavage figures in *Chaetopterus* eggs, after fixation in Boveri's reagent at various hydrogen ion concentrations. The slide-making procedure was standard. *Result:* Central body structure is related to ray structure; changes occur in both as the pH is varied.

Experiment 8. The Effects of Varying the Temperature of Boveri's Reagent

Eggs from a single egg-set were fixed in two samples of Boveri's picro-acetic fluid, one chilled to 1°C., the other heated to 95°C. These temperatures were of course slightly modified when the eggs and the ever-present sea-water were added, as the latter were at 21° C.

When the reagent is at 1°C., the same two types (1*B* and 2*B*) occur in about the same proportions as when the eggs are fixed under ordinary conditions at room temperature. But at 95°C. all asters have vague rippled rays and disrupted centers (1*D*). In other words, chilling the fixative produces no deviation from the usual condition, but heating it causes changes (Chart 5). The relation between ray structure and central body structure is once more apparent.

Experiment 9. The Effects of Varying the Duration of Fixation with Boveri's Reagent

Eggs were fixed in Boveri's picro-acetic reagent for periods of time varying from one second to six months, as shown in Chart 6. This

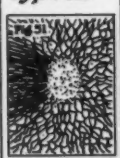
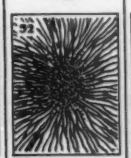
EXPERIMENTAL DATA		DISTRIBUTION of ASTRAL TYPES at each TEMPERATURE		
		Type 1B	2B	1D
Temperature of Boveri's Reagent at time of Fixation	Number of cells studied			
1° C	31	19 61%	12 39%	
95° C	30			30 100%

CHART 5. THE EFFECTS OF VARYING THE TEMPERATURE OF BOVERI'S REAGENT

Central bodies were studied in metaphase first-cleavage figures in *Chaetopterus* eggs, after fixation in Boveri's reagent at 1° C. and at 95° C. The slide-making procedure was standard. *Result:* Central body structure is related to ray structure. At low temperatures the central bodies and ray configurations are the same as those occurring at ordinary temperatures; at high temperatures the situation is modified.

experiment shows the speed at which the reagent penetrates and fixes the eggs and also the effects of duration of fixation upon astral structure.

The experimental set-up employed when fixing eggs for periods as brief as one or several seconds was as follows: in order to effect instantaneous mixing, two drops of a thick egg suspension were suddenly squirted into 2 cc. of the reagent, which was kept in motion in a small

Stender dish. This proportion of eggs to reagent weakens the fixative so slightly that any effects of dilution can safely be ignored. After the desired interval of fixation, one or more seconds, the entire mixture was poured into 400 cc. of 70 per cent alcohol, the fluid always used to wash material fixed with a picro-acetic reagent. The addition of 2 cc. of eggs and fixative to 400 cc. of alcohol dilutes the fixative so effectively as to stop its further action. However, to make sure that the alcohol itself, plus the limited amount of reagent added with the eggs, did not modify the coagulation product, a control experiment was run, in which eggs were fixed in 70 per cent alcohol containing 0.5 per cent picro-acetic reagent. The asters in these eggs show only vague rays, and central areas entirely undifferentiated from the peripheral part (*2D*, Fig. 57). Hence the washing in alcohol can be dismissed as a factor in producing the types of astral structure occurring in the experiment. The set-up for fixing eggs for longer periods of time was the usual one.[12]

This experiment shows, first, that complete fixation of the eggs in full strength picro-acetic reagent occurs within one second; (the speed of fixation in dilute reagents is not known). In the second place, it shows that different lengths of exposure to the reagent modify the types of central bodies present. These differences, at the various periods of fixation, are as follows:

Fixation time 1, 3, or 6 seconds: All asters have medium coarse undulating rays; 28 of the 34 centers studied are even (*2B*); the remaining 6 are dense (*3B*).

Fixation time 30 seconds: 35 per cent of the asters have disrupted centers and medium coarse rippled rays (*1B*), and 65 per cent have even centers and medium coarse undulating rays (*2B*). These are the same types which occur after the usual periods of fixation for hours, but the proportions in which they occur are intermediate between those of eggs fixed but a few seconds, when there are no disrupted centers, and those occurring under usual conditions, when there is a high percentage of disrupted centers.

Fixation time 10 minutes or fifteen hours: 90 per cent of the asters have disrupted centers (*1B*), and 10 per cent have even ones (*2B*).

Fixation time thirteen days: only 28 per cent of these eggs are of the disrupted type, 72 per cent having evenly filled centers. The percentages of both types occurring in this set are again intermediate, this

[12] The samples of eggs at the three shortest intervals are intentionally small, because of the effort to keep the egg mass as small as possible. The equally small samples reported for the sets fixed at longer intervals are explained by the fact that this particular batch was fixed a little too early, due to a miscalculation, and but few of the eggs had reached metaphase. However, in spite of the small size of the samples and the error necessarily involved, the major facts are clear.

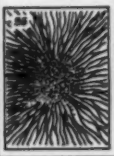
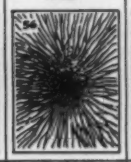
EXPERIMENTAL DATA		DISTRIBUTION OF ASTRAL TYPES AT EACH DURATION of FIXATION			
		Type 1B	2B	3B	2D
Duration of fixation in Boveri's Reagent	Number of Cells studied				
1 second.	*15*		*11* 73%	*4* 27%	
3 seconds.	*10*		*9* 90%	*1* 10%	
6 seconds.	*9*		*8* 89%	*1* 11%	
30 seconds.	*20*	*7* 35%	*13* 65%		
10 minutes.	*20*	*18* 90%	*2* 10%		
15 hours.	*20*	*18* 90%	*2* 10%		
13 days.	*18*	*5* 28%	*13* 72%		
6 months.	*21*		*21* 100%		
CONTROL:- Eggs fixed in 70% Alcohol containing 0.5% Boveri's Reagent.	*20*				*20* 100%

CHART 6. THE EFFECTS OF VARYING THE DURATION OF FIXATION WITH BOVERI'S REAGENT

Central bodies were studied in metaphase first-cleavage figures in *Chaetopterus* eggs, after fixation in Boveri's reagent for various periods of time. The slide-making procedure was standard. *Result:* Central body structure is related to ray structure. Fixation for a few seconds produces only even central bodies and undulating rays; fixation for hours produces in most cases disrupted centers and rippled rays; fixation for months produces the same result as fixation for seconds.

time between those of eggs fixed for hours and those fixed for months.

Fixation time six months: all asters have even centers and undulating rays (*2B*).

In brief, this experiment shows that fixation for a few seconds produces only asters with filled centers, even or dense, and undulating rays; fixation for minutes and hours produces a majority of asters with disrupted centers and rippled rays and a minority with filled centers and undulating rays; and fixation for months returns the aster to the same condition as that produced by fixation for a few seconds. The major result established in the previous experiments is reaffirmed here: ray structure and central body structure are related.

In all of the experiments reported in this paper except this one, the eggs were left in the reagent from six to fifteen hours. This period of fixation falls within the limits of the groups fixed in this experiment at ten minutes and fifteen hours, during which time no modification of central body structure occurred as a result of the length of exposure to the reagent. Hence it can be assumed that they were not affected by differences in length of exposure to the fixative.

IV. Discussion

All of the astral types previously described are arranged in Chart 7, where they are classified on a three-fold basis:

(A) According to differences in structure there are five classes of central bodies:

Number and Name	*Structure*
1. Disrupted.	Not filled. Entirely empty or containing scattered material.
2. Even.	Filled. Not demarked from ray area. Stained like ray area.
3. Dense.	Filled. Not demarked from ray area. Stained darker than ray area.
4. Slightly demarked, without centriole.	Filled. Slightly demarked from ray area. Stained darker than ray area.
5. Slightly demarked, with single or double centriole.	Filled. Slightly demarked from ray area. Stained darker than ray area.

(B) In shape, the rays take two forms: first, rippled or serpentine, and second, undulating or almost straight.[13] Members of the intermediate, or slightly rippled class are included with the other two (p. 156).

(C) On the basis of coarseness there are five classes of rays: (1) very coarse, (2) medium coarse, (3) delicate, (4) vague, and (5) absent.

[13] See footnote 4, p. 155.

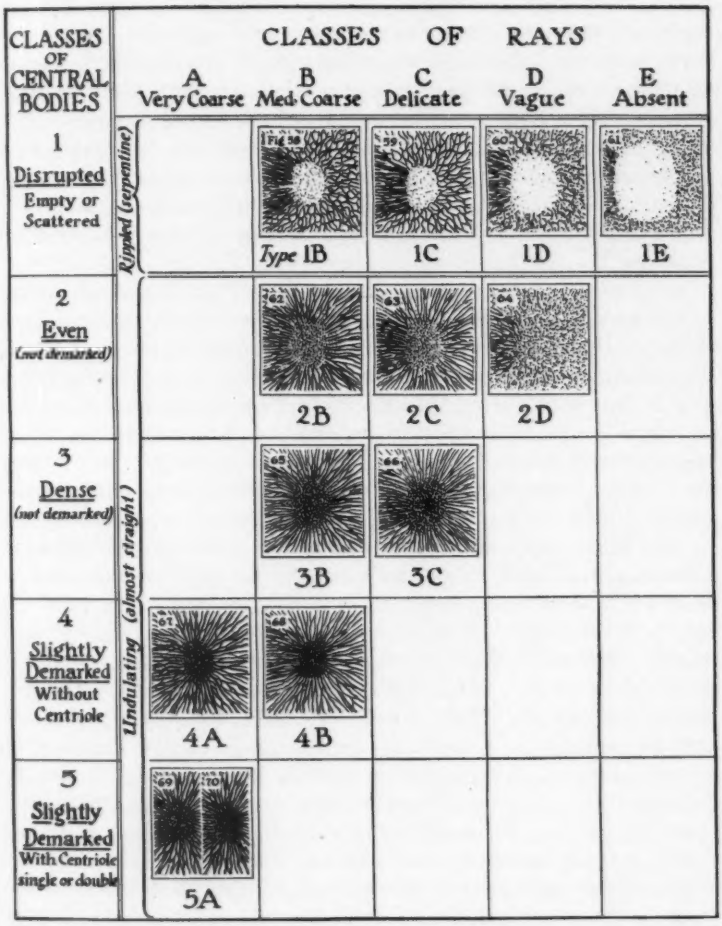


CHART 7. THE ASTRAL TYPES OCCURRING AFTER PICRO-ACETIC FIXATION

Central bodies were studied in metaphase first-cleavage figures in *Chaetopterus* eggs, after fixation with different modifications of a picro-acetic reagent and various changes in the slide-making procedure. *Result:* Central body structure is related to ray structure. If rays are rippled, centers are disrupted; if rays are undulating, but delicate or medium coarse, centers are usually even (or dense) centrosomes, undemarked from the ray area; only if rays are undulating and also very coarse is there a vaguely demarked centrosome containing a centriole, single or double. These occur in 58 per cent of the cases, and they vary in size and shape.

The following facts are apparently established by this study, in metaphase first-cleavage figures of *Chaetopterus* eggs:

I. The Astral Center is Very Unstable.—The chromosomes maintain their usual form under many modifications of technique, except under a few conditions such as extreme dilution of the reagent, or change in the pH. The spindle, too, is modified only occasionally, and its fibers are affected much less than the astral rays. The aster as a whole, although varying in size, is usually clearly demonstrated. The detailed structure of center and rays, however, shows maximum instability.

II. The Variations in the Structure of the Astral Center are Related to Variations in Ray Structure.—(1) If the ray area is completely nonradial or shows but a vague radial organization, the center is either disrupted (1*D* and 1*E*) or undifferentiated from the ray region (2*D*). (2) If the rays are fixed distinctly and are rippled in shape, the center is never filled; it is always disrupted (empty or scattered), regardless of whether rays are delicate (1*C*) or medium coarse (1*B*). These asters, whose centers are either entirely undifferentiated from the ray area or broken and disrupted, will not be discussed further at this time.

(3) If the rays are fixed distinctly and are undulating in shape, the center is always filled, its detailed structure varying with the coarseness of the rays in several respects: (*a*) Size: the coarser the rays, the smaller is the center. When rays are delicate or medium coarse most centers are about $5 \times 6\,\mu$ in size; when rays are very coarse centers are about $4 \times 5\,\mu$ in size. The latter frequently contain minute centrioles. (*b*) Depth of stain: the coarser the rays, the more darkly does the center stain. When rays are delicate or medium coarse, most centers are even, and stain like the ray area (2*B* and 2*C*); occasionally they are dense, staining somewhat more deeply (3*B*, 3*C*, 4*B*). But when rays are very coarse the center always stains darker than the ray area.[14] (*c*) Demarcation from ray area: the coarser the rays, the greater is the tendency for the center to be demarked from the ray region. When rays are delicate or medium coarse, centers are usually not demarked from the ray area, but gradually merge with it (2*B*, 2*C*, 3*B*, and 3*C*); but when rays are very coarse the center (centrosome) is usually vaguely demarked from the ray region (4*A* and 5A). The degree of demarcation varies not only from center to center, but in

[14] The difference in appearance between the even (2*B* and 2*C*) and the dense (3*B* and 3*C*) types of centers may be due to nothing but slight differences in the depth of stain. This factor, however, does not account for the dark stain of the centers (centrosomes) in asters having very coarse rays (4*A* and 5*A*). These are deeply stained even when the slide is stained very lightly; in fact such slides must be lightly stained, or the centers are clogged with dye.

different parts of the same center; there is no suggestion of a limiting membrane. (*d*) Presence of centriole: only when rays are very coarse does the center show a double zone of differentiation. When rays are delicate or medium coarse, and also in 42 per cent of the asters with very coarse rays, the center is comprised of but a single zone, the centrosome (*2B, 2C, 3B, 3C, 4A* and *4B*). But in 58 per cent of the asters with very coarse rays the center has two zones of differentiation, the centrosome and the centriole (*5A*).

In short, the center behaves more like an area of variable size and form than like a small individualized body of definite size. The coarser and straighter the rays are, the more condensed is the center—*i.e.*, it is smaller, darker, and more delimited. The twelve central body types occurring in the study constitute a graded series, from a completely empty area at one extreme, to a minute centriole surrounded by a vaguely demarked centrosome at the other.

III. When Centrioles are Present They Show Variations in Structure.—Of the 207 asters studied which have very coarse undulating rays and vaguely demarked centrosomes (*4A* and *5A*: Chart 2, Figs. 11 to 13, representing 187 cases; Chart 3, Fig. 35, representing 20 cases), only 121, or 58 per cent, have centrioles. These have rough and irregular edges and they vary in shape and size. Their configuration in the large aster bears no relation to that of the small one of the same mitotic figure.

IV. The Centriole can be Demonstrated Only under Very Narrow Conditions of Fixation.—To show a centriole when using a picro-acetic mixture, the picric acid must be present in amounts between about 0.01 per cent and 0.2 per cent by weight and the acetic between about 0.05 per cent and 0.4 per cent; all other concentrations used in the present study produce centers without centrioles and rays which are never coarse and undulating. When either or both of the acids are present in very small amounts (Figs. 16, 25, and 42, Chart 3) it is understandable that the reagent may fail properly to coagulate the aster and may thus give meaningless results; but once a concentration is reached which produces very coarse undulating rays and a centriole, it is reasonable to expect that some increase in the amount of either acid would still produce the same kind of coagulation product. Instead of this, however, as soon as the concentration of either exceeds that of the critical zone even slightly, both rays and central bodies are modified. The next paper on *Chaetopterus* eggs will show that equally restricted conditions govern the demonstration of centrioles when diverse reagents are used, both full strength and diluted.

Turning now to the interpretation of these data, it is apparent that the centriole in *Chaetopterus* eggs at metaphase of first cleavage does not behave like a typical centriole, such as is frequently found during the spermatogenesis of many species. The demonstration of the former is dependent upon the simultaneous demonstration of a specific type of ray structure; the latter can be demonstrated whether the mitotic figure is present or absent. The former can be shown only if the fixing agents are present within exceedingly narrow limits of concentration, and even then exhibits variations in size and shape; the latter is readily fixed despite considerable variations in the concentrations of the chemical components of diverse reagents, and shows a similar size and shape in practically all cases. If we are dealing with a true centriole in *Chaetopterus* eggs it is necessary to select one of the twelve centers comprising a continuous series, and regard it as "well fixed," assuming that it is a cell component so constituted chemically that it can be coagulated only under certain narrow conditions, and to dismiss the other eleven types, some of which occur on the same slide, as "poorly fixed."

This behavior of the *Chaetopterus* centriole is not unique. A similar relationship has been established between the structure of central bodies and the structure of rays in cytasters, sperm asters, and first-cleavage figures in *Echinarachnius* eggs (Fry, 1928, etc.) Other studies, soon to be published, demonstrate the existence of a close relation between central body morphology and ray structure in different cell types of various species. Naturally the details differ in each case. In these cells the centrioles exhibit an instability of structure and a relation to ray configuration not generally recognized.

Another and quite different interpretation of the nature of centrioles in *Chaetopterus* eggs is suggested by the data of the present investigation. We may be dealing here, not with an individualized body which exists in the living egg, but with a coagulation product of the focal area of astral rays and spindle fibers. The aster is universally recognized as a complex radial system of converging ray and inter-ray materials, in which the inter-ray substances are crowded out near the center. Since this leaves the central area composed largely or entirely of ray materials, differing both chemically and physically from the peripheral region, is it not possible that this focal area may coagulate differently from the outer parts? Furthermore, its size and physical structure might depend upon the way in which the radial organization of the aster is coagulated —*i.e.*, whether the fixed rays are coarse or delicate, undulating or rippled. Fixation changes in the peripheral portion of the aster may

be related to changes at the center. In such an event, one or more of the configurations produced at the center as coagulation products of the focalized rays and spindle fibers might be small, dark, and delimited, and thus simulate an individualized body. In other words, the demonstration of a small demarked body at the astral center may not necessarily prove the existence of a centriole.

On the basis of this interpretation, the centriole in metaphase first-cleavage figures of *Chaetopterus* eggs is in all probability a focal coagulation artifact. Further consideration of this possibility will be held in abeyance until more data are available in this and other species. In the meantime, in this and future papers, bodies behaving as do *Chaetopterus* centrioles will be spoken of as " focal bodies," to indicate the fact that their structure has a close relation to the detailed structure of focalized rays and fibers, in contrast to that of a typical centriole, which is independent of the exact configuration of rays and fibers.[15]

Some of the confusion in the central body problem may be caused by the possibility that the minute bodies which occur at astral centers actually may belong to several categories, such as the following:

(1) *Random Granules.*—At the astral center of certain cells there are present, after a given fixation, various granules like those in the cytoplasm, which differ in size, number, and location. In *Chaetopterus* eggs they occur very rarely, in the even type of central body (*2B*; discussion p. 157). In *Asterias* eggs they are abundant during the first maturation division after Bouin's fixation. They are likewise abundant in telophase first-cleavage figures of *Cerebratulus* eggs after picro-acetic fixation. This evidence has not yet been reported. Where such granules occur, their configurations in certain asters simulate centrioles, either single or double. Such granules have occasionally been interpreted as centrioles in the past, but it is a simple matter to prove that such configurations are but members of a series.

(2) *Focal Bodies.*—To this group belong the central bodies in *Chaetopterus* eggs described in the present study, central bodies in *Echin-*

[15] The fact that the metaphase centriole of *Chaetopterus* eggs, when present, is either single or double, seems to indicate that it is undergoing division. If such is the case, this is conclusive evidence that we are dealing with a self-perpetuating individualized cell component. But this doubling can also be satisfactorily explained in a way which is in harmony with the suggestion that these structures are focal bodies. However, this question cannot be adequately considered in an investigation which confines itself to metaphase. It will be fully discussed in a later report on first cleavage in *Chaetopterus* eggs, in which centrioles are studied from prophase to telophase, after fixation with reagents which demonstrate them with maximum clearness.

arachnius eggs, and in various other cells to be described in later papers. The morphology of focal bodies is closely related to the exact structure of focalized rays and spindle fibers. In the absence of asters, focal bodies may exist at the spindle-tip, if the latter is sharply focused. If it is blunt, however, as in certain anastral polar-body figures, focal bodies are absent. They are never present before the areas of focalized rays or fibers are formed, and they always vanish when such areas disintegrate, although the outer region of the asters and the middle area of the spindle may still persist. Their behavior differs from that of typical centrioles which exist independently of focalized rays and fibers. Future study may show that certain centrioles which have been assumed to be typical, such as in *Chaetopterus* eggs (Mead, 1898, Wilson, 1930), actually are focal bodies. It remains to be proved, however, whether such focal bodies are individualized structures existing in the living cell, and are unusually difficult to demonstrate, or whether they are merely coagulation artifacts of focalized rays and fibers. In any event their behavior differs so markedly from that of typical centrioles that they should not be confused with them.

(3) *Focal Staining Artifacts.*—These phenomena, described long ago by Fischer (1899) and others, are due to the fact that some asters and spindle-tips, after certain types of fixation, hold the dye with great tenacity at the focal area. In such cases continued destaining makes the body progressively smaller, and finally results in its disappearance. At a certain stage in the process, however, there appears to be a centriole, its size depending upon the degree of destaining. In contrast to such a staining artifact, a focal body exists in the coagulation product as a non-radial body at the astral center, which, if large enough, can be seen in sectioned and unstained material; in other forms, where they are smaller, and when unstained are invisible with white light, they can be demonstrated by ultra-violet photography. A typical staining artifact, on the other hand, contains no demonstrable body at the focal area; instead, the rays or fibers reach the center of the area, and progressive destaining changes the appearance of the center in contour, size, and other points. In the focal body, wide differences in the degree of destaining, short of extreme over- or under-staining, have no effect on its size, since it exists as a non-radial structure at the astral center, and holds the dye somewhat differently than does the surrounding radial area. In some cells the situation is complicated by the fact that both phenomena may be involved: there may be minute focal bodies, in addition to which overstaining may produce staining artifacts in the inner zone of surrounding fibers. These points will be developed in later papers.

(4) *Centrioles.*—These are stable structures which, unlike focal bodies, maintain their characteristic size and shape in spite of extensive modifications in the coarseness and shape of the rays and fibers. In some cells they maintain genetic continuity as individualized bodies from one cell cycle to the next, whether the mitotic figure is present or absent, as in *Drosophila* eggs (Wilson and Huettner, 1931) and in *Amphiuma* leucocytes (Pollister, 1932); in other cases they may disappear during interkinesis, but when present they exhibit a stability of structure unlike that of focal bodies. They are readily demonstrated in their characteristic form by a variety of reagents.

(5) *Blepharoplast-centrioles.*—These are found only in motile cells, or in the forerunners of such cells, with the exception of certain atypical ones, such as the aflagellate sperms of *Ascaris,* recently reexamined by Sturdivant (1931). They have been studied most extensively in the spermatogenesis of animals, where they may arise in the primary spermatocyte or earlier; or they may not appear until the spermatid stage. Like the centrioles of non-motile cells, they are exceedingly stable and show their characteristic morphology when fixed by many different reagents; they are unaffected by changes in the physical structure of focalized rays or fibers; and in some species they are present in the entire absence of such areas. In some cells blepharoplast-centrioles are visible in the living condition. These centrioles are ordinarily thought of as in the same class as those of non-motile cells. This may actually be the case, but the fact that the one is a blepharoplast as well as a centriole, while the other is only a centriole, makes it advisable to give them separate terms which suggest this distinction in their behavior.

(6) *Other Categories.*—There are other types of structures which have been called central bodies such as the following: (*a*) *Bi-lobed bodies of sperm.* In the middle-piece of the echinoderm sperm is a bi-lobed structure of chondriosome material which Boveri misinterpreted as centrioles. That such is not the case was long ago shown by various workers.[16] (*b*) *Erythrocyte centrosomes.* Structures are present during the vegetative phase of erythrocytes in various species which have been interpreted as central bodies. Dawson (1932) has shown that such an interpretation is not valid. (*c*) *Diplosomes of epithelial cells.* Bi-lobed structures lying near the outer part of certain epithelial cells have been identified as central bodies. Whether or not such is actually the case remains to be proved.

Centrioles are generally considered to be the most stable and persistent component of the mitotic figure in the animal cell, and are assumed to play some kind of a formative rôle in connection with the

[16] For a discussion of this see Fry, 1929*a*, p. 105.

origin of asters and spindles, in some cases giving genetic continuity to successive division figures. It is therefore important that we ascertain whether the cell structures we now call centrioles belong to one category or to several, and that we determine the inter-relationships of these classes. Before this can be done, however, the centrioles of many diverse types of cells must be studied, keeping in mind the various modes of behavior, and also the dangers of misinterpreting certain structures that look like typical centrioles but actually may not be so. In the meantime we must be cautious in generalizing from the behavior of one class to that of the others.

V. Summary

In earlier studies of *Echinarachnius* eggs the writer reached the conclusion that their central bodies are the coagulated focal area of converging rays and have no existence as individualized structures in the living cell. The present investigation is the first of a group analyzing central body behavior in a variety of cell types in diverse organisms, for the purpose of ascertaining in each case whether the central body behaves like that of *Echinarachnius* eggs or like a typical centriole, such as that found in many spermatocytes.

The present study confines itself to first-cleavage metaphase asters in *Chaetopterus* eggs, because, in preliminary experiments, the writer failed to find the typical centrioles of that egg previously described by Mead, whose observations were later confirmed by Wilson. The effects upon central body structure produced by the following modifications of technique were observed: the formula, pH, and temperature of the reagent, the duration of fixation, the depth of stain, and other points.

Ray structure is readily modified in coarseness; the shape is also affected, the rays being either rippled (serpentine) or undulating (almost straight). Central body structure is closely related to ray structure: if rays are rippled, centers are disrupted; if rays are undulating but delicate or medium coarse, centers are large homogeneous centrosomes, undemarked from the ray area; only if rays are both undulating and very coarse is there a slightly demarked centrosome, which, in 58 per cent of the cases, contains single or double centrioles, which vary in size and shape.

Centrioles can be demonstrated only under a very limited set of chemical conditions. Among the many modifications of the formula employed, the only ones coagulating centrioles and very coarse undulating rays are those in which the reagent was diluted with about 80

parts of water. In these the percentage by weight of the picric acid is between about 0.01 per cent and 0.2 per cent, and of the acetic acid, between about 0.05 per cent and 0.4 per cent. Any deviations from these conditions produce other types of both centers and rays.

If the central body in *Chaetopterus* eggs is a true centriole, it is atypical in several respects: the great instability of the central body area, the close relation between its morphology and the exact configuration of the fixed rays, and the narrowly limited conditions of fixation under which centrioles can be shown.

The suggestion is made that, as in *Echinarachnius* eggs, the various kinds of central bodies occurring may be various types of coagulation products of the focal area of converging rays, the morphology of the center depending upon the way in which the rays are fixed; only by chance do certain of them simulate individualized structures. Further consideration of this point awaits additional data.

Various classes of minute bodies occurring at the centers of fixed asters are discussed, and the suggestion is made that heretofore we have applied the single term centriole to several very diverse classes of structures, which often look alike but actually behave so differently as to warrant a careful consideration of whether or not they belong to several different categories.

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EXPERIMENTAL PRODUCTION OF CHAINS AND ITS GENETIC CONSEQUENCES IN THE CILIATE PROTOZOAN, COLPIDIUM CAMPYLUM (STOKES)

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I. INTRODUCTION

In 1925, Édouard and Mme. Chatton showed that chains of individuals could be produced in *Colpidium campylum* and *Colpidium colpoda* by feeding them a certain strain of the bacterium *Bacillus coli* (*"Colibacillus D"*). The present paper deals with the genetic consequences of a similar chain-formation induced in *Colpidium campylum* [2] by a strain of another bacterium (*Micrococcus sp.*, probably *sensibilis*). The present work is in part an independent corroboration of the work of the Chattons, because the author was unacquainted with their work until the essential points in which the two investigations agree had already been established.

In the present work, though not in the work of the Chattons, experimentally produced chains gave rise to a new racial type,—double animals. As a result of this, the question in the foreground of interest

[1] During the performance of most of the experimental work, the author was Fellow of the National Research Council. The author wishes to acknowledge his indebtedness to Professor H. S. Jennings for many important suggestions, particularly concerning preparation of the manuscript; and to Dr. Ruth S. Lynch for critical reading of the manuscript.

[2] Identified through the courtesy of Professor Édouard Chatton.

in this paper is: What are the genetic consequences of the experimental production of chains? In the treatment of this question, the following matters will be taken up: (1) the experimental determination of chain-formation; (2) the mode of origin of chains and their subsequent development into races of doubles; (3) the persistence and stability of the races of doubles; (4) the existence of diverse biotypes among doubles and their descendants; (5) an examination of the nature of the processes involved in these changes of hereditary characteristics in vegetative reproduction.

II. Materials and Methods

The methods employed for the cultivation of large mass cultures of *Colpidium* were given in an earlier paper (Sonneborn, 1930*a*).[3] The basic fluid was an infusion of 1.5 grams rye grains boiled for ten minutes in 100 cc. spring water, filtered and let stand for 24 hours to "ripen." Two hundred cc. of this infusion plus five boiled rye grains were put into a finger bowl 10 cm. in diameter and 4 cm. deep. This was inoculated with 10 to 15 cc. of an old culture of *C. campylum;* within 24 hours it would develop into a flourishing culture and remain so for several days. When the colpidia began to get smaller and paler, usually in two to six days, subcultures were made in the same way. Only large stock cultures were maintained in this way.

Smaller mass cultures were kept in square salt cellars and in Columbia dishes. These were begun with several drops of ripe fluid (without any rye grains) and ten to thirty colpidia; each day more ripe fluid was added until after three to five days the dish was full (about thirty drops). Subcultures were then made in the same way.

In special experiments designed to investigate carefully the factors determining chain-formation, Columbia dish cultures were made in a different way, following, in many respects, the bacteriological technique employed by Raffel (1930) in the cultivation of *Paramecium.* The standard rye infusion, as soon as filtered, was distributed in cotton-plugged test tubes, autoclaved and stored until needed. When needed, a tube was opened over a Bunsen flame and inoculated by means of a platinum needle with a pure culture of either *Achromobacter sp.,* probably *candicans,* or *Micrococcus sp.,* probably *sensibilis,* grown on beef-agar slants. The inoculated culture fluid was then pipetted into Columbia dishes, inside of Petri dishes. These, and the cotton-plugged pipettes, inside of jars, had all been heated for one hour at 150° C. in a hot-air sterilizer. Each pipette was used only once and the top of the Petri dish containing the Columbia dish was raised only enough to admit

[3] In that paper, the species was incorrectly called *Colpidium striatum.*

the pipette with culture fluid and colpidia. The colpidia had previously been washed according to the method of Parpart (1928). After such a culture was set up, it was opened only once for purposes of subculture.

In all the rest of the work, the colpidia were cultured in isolation on ground glass slides containing two concavities. Twelve of these slides were placed on a glass plate raised on glass supports in an inverted 9-inch Petri dish sealed with water at the bottom. The "ripe" rye infusion was used as cultivation medium, one drop to each concavity. Each day one *Colpidium* was placed in such a drop; 24 hours later the drop was again observed, records of reproduction and other matters of interest were made, and one of the colpidia was transferred to a fresh drop of ripe culture fluid. Each day a similar procedure was followed.

A number of other details of procedure in the isolation cultures are important: (1) The possibility of perpetuating, by daily transfers, differences in bacterial flora between different lines was avoided by collecting a small amount of fluid from each 24-hour-old culture drop and using these small drops for inoculating the culture fluid for the next day. Such cross-inoculations were performed daily in some experiments, less frequently in others. (2) The possibility of repeating daily systematic differences in the treatment of the lines compared was avoided by the following methods: (a) within each moist chamber containing 24 lines, each type of animal in a particular set of comparisons was represented by the same number of lines; (b) within each moist chamber the lines were distributed according to a plan whereby the lines of the same type or race were separated from each other and whereby the arrangement in no two moist chambers was the same; (c) the order in which the moist chambers were transferred was systematically changed from day to day. (3) The possibility of personal bias influencing the results was avoided by assigning to each line for daily identification a name that gave no indication of its genetic history. The key to these names was not consulted until the end of the experiment.

Many of the experiments were conducted at room temperature of 20°–25° C., some were run in a constant temperature chamber which only rarely exceeded the range 22°–23° C. The animals used in all the work here reported were descended from one individual and were never known to conjugate, even when efforts were made to make them do so.

III. The Determination of Chain-Formation

As already mentioned, Édouard and Mme. Chatton were able to induce the formation of chains in *Colpidium campylum* and *Colpidium colpoda* by feeding these species a particular strain of *Bacillus coli.* Other species of bacteria, other strains of *Bacillus coli,* and this strain

grown on other than a vegetable base did not induce chain-formation in *Colpidium*. These investigators thus demonstrated that the formation of chains in *Colpidium* is determined by a particular strain of bacteria grown on a vegetable base.

This result of the Chattons was confirmed by the present investigation, as will now be set forth. The rye infusion in which chains of colpidia had arisen was plated out on agar (with the help of Dr. Raffel) and the different kinds of bacteria were separately cultivated. Only two kinds of colonies could be distinguished: one yellow and rapidly growing, the other white and slowly growing. These two species were identified for me through the courtesy of Professor William W. Ford of the Department of Bacteriology, School of Hygiene and Public Health, the Johns Hopkins University. The yellow species was identified as belonging to the genus *Micrococcus* (Bergey) and corresponding closely to the species *M. sensibilis;* the white species was identified as belonging to the genus *Achromobacter* (Bergey) and corresponding closely to the species *A. candicans.*

A number of experiments indicated an increase in the frequency of chain-formation when *Micrococcus* predominated in the food supply. In a few cultures some chains were formed in fluid in which *Achromobacter* predominated; in these cultures, however, *Micrococcus* had not been excluded. In all other cultures in which chains were formed, *Micrococcus* had definitely been inoculated into the culture fluid.

Critical experiments, using the rigorous bacteriological methods described above in Section II, were performed to discover the relation of these two species of bacteria to chain-formation. On July 29, eight normal colpidia were washed, according to Parpart's (1928) method, in two steps: five washings were followed by a lapse of five hours, after which five more washings were made. Four of the eight colpidia were washed in autoclaved rye fluid inoculated with *Achromobacter;* the other four in similar fluid inoculated with *Micrococcus.* By July 31, each set of four had multiplied to form eighteen colpidia. The animals in *Micrococcus* fluid were used to establish nine cultures, each consisting of two colpidia in sixteen drops of autoclaved rye fluid inoculated with *Micrococcus.* The animals in *Achromobacter* were used to establish nine similar cultures in *Achromobacter* fluid. Counts of the number of singles and the number of chains in each of these eighteen cultures were made on August 3. As appears in Table I, not one chain was formed among 46,716 colpidia produced in the pure *Achromobacter* fluid, but 51 chains were formed among 11,912 colpidia produced in the pure *Micrococcus* fluid. The production of chains is clearly deter-

TABLE I

Comparison of the percentage of chains formed in cultures fed Micrococcus exclusively, with the percentage formed in cultures fed Achromobacter exclusively

BACTERIUM SUPPLIED		CULTURES									
		1	2	3	4	5	6	7	8	9	Total
Achromobacter	Number of Colpidia	5765	6720	5741	4849	5186	5815	4240	4852	3548	46716
	Number of Chains	0	0	0	0	0	0	0	0	0	0
	Percentage of Chains	0	0	0	0	0	0	0	0	0	0
Micrococcus	Number of Colpidia	1779	1184	1679	1354	1576	1071	1336	1224	1709	11912
	Number of Chains	21	1	1	5	2	0	6	0	15	51
	Percentage of Chains	1.18	0.08	0.06	0.37	0.13	0	0.45	0	0.88	0.43

mined by the presence of the *Micrococcus*. However, only a small proportion of the colpidia subjected to *Micrococcus* form chains. This is in striking contrast to the result reported by the Chattons with "*Colibacillus D*." In their work, all dividing colpidia formed chains in this bacterium. The relatively small proportion of chains produced with *Micrococcus* may have been due to the fact that this bacterium was cultivated on beef agar before being introduced into the rye fluid with *Colpidium*. The Chattons, it will be recalled, showed that the chain-inducing power of *Colibacillus D* was lost when it was grown on other than vegetable bases.

In the present work the existence of additional factors in the determination of chain-formation was indicated by the considerable variation in percentage of chains formed among the nine cultures reared on pure *Micrococcus*. One of these factors may be the concentration of colpidia in the culture fluid. Although all cultures began with two colpidia in sixteen drops, there was much variation in their concentration at the end of the experiment. The mean number of animals per micropipette drop varied from 48.7 to 84.7 colpidia. The five cultures in which there were more than 70 colpidia per droplet produced, on the average, 0.54 per cent chains; the four cultures in which there were less than 60 colpidia per drop produced, on the average, 0.11 per cent chains. There is, therefore, some indication that the percentage of chains formed when fed *Micrococcus* depends to some extent on the concentration of colpidia in the fluid.

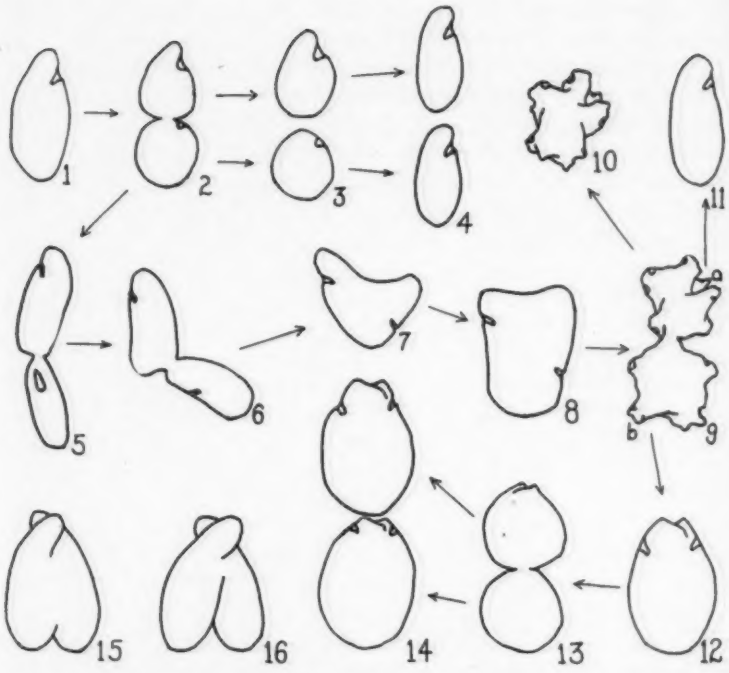
The essential point, however, is that the difference between cultures which remain normal and cultures which produce chains is due to the difference between the effects of two diets: cultures fed only *Achromobacter* remain normal; cultures fed *Micrococcus* produce a small percentage of chains. This dependence of chain-formation in *Colpidium* upon the presence of particular strains of bacteria confirms the earlier results of the Chattons. The latter investigators attempted also to induce chain-formation in other genera of ciliates with the strain of bacteria they found effective for *Colpidium*. Such attempts failed completely. The present investigator also attempted to induce chains in *Paramecium aurelia*, by feeding this species the strain of *Micrococcus* found to be effective for *Colpidium*. Like the attempts of the Chattons, this attempt also failed. It appears that *Colpidium* is a genus particularly susceptible to this type of bacterial action.

IV. Origin of Chains and Their Subsequent Development

Chains originate simply as a consequence of the development of the adult form in the two parts of a dividing individual, in the absence of

the normal separation of these two parts. A normal adult (Fig. 1) becomes constricted transversely in about the mid-region (Fig. 2). In normal fission, separation of the two parts occurs at this stage (Fig. 3), and each part develops the adult form (Fig. 4). In chain-formation, the two parts attain the adult form while still united (Fig. 5), and separation of the parts is either much delayed or permanently suppressed. In normal fission, the processes of transverse constriction and separation of parts take about twenty minutes; in chain-formation, the two parts remain together several hours or permanently. The parts of the chains usually remained united several hours in the work of the Chattons, but the union was permanent in most, if not all, of the chains produced in the present work. The Chattons also found that some chains developed into three or four parts as a consequence of a second incompleted fission, and that all chains possessed elongated alimentary vacuoles. In the present work, the alimentary vacuoles of chains retained the normal spherical shape and no chains of three or four parts were observed. In all chains isolated for observation, the junction between the two parts widened and their aboral surfaces gradually bent more and more towards each other (Fig. 6). This process continued, with increasing fusion of the two aboral surfaces (Fig. 7), until their entire lengths were completely fused (Fig. 8). Such individuals thus contained two complete sets of internal structures arranged along parallel axes, but with opposite polarity: the former anterior aboral surface of each part was fused to the former posterior aboral surface of the other part. From such heteropolar double animals, multiple monsters of irregular form (Fig. 9) arose as a consequence of further incomplete fissions: sets of internal structures (nuclei, oral grooves, etc.) multiplied, but the new sets did not separate as new individuals. The usual fate of such irregular multiple monsters was either to die or to break up into smaller irregular monsters (Fig. 10). Occasionally, however, the multiple monsters produced apparently normal individuals (Fig. 11). These gave rise to normal descendants. Less frequently, multiple monsters produced double individuals (Fig. 12) with their two sets of internal structures having identical polarity. The identical polarity of the two parts of these doubles is in contrast to the polarity of the doubles (Fig. 8) earlier formed. The production of such homopolar doubles from multiple monsters was not reported by the Chattons.

The probable mode of origin of normals and homopolar doubles from multiple monsters was indicated by the structure of the monsters. Occasionally, a projection on a monster contained a single set of structures (Fig. 9*a*); fission across such a projection would yield an indi-



FIGS. 1–16. The normal division cycle and the origin of doubles from chains and from "pseudo-conjugants." Figs. 1, 4, 5, 6, 7, 8, 11, 12, 13, and 14 from camera lucida drawings; Figs. 2, 3, 15, and 16 from notebook sketches; Figs. 9 and 10 diagrammatic. Figs. 1 and 11, normal adults; Fig. 2, normal fission nearly completed; Fig. 3, fission just completed; Fig. 4, recent products of fission that have already attained adult form; Fig. 5, chains of two parts, each part having the elongated adult form; Fig. 6, chain with the two parts united over a wider region and forming an obtuse angle; Fig. 7, chain with the two parts united over a large part of their aboral surfaces and forming an acute angle; Fig. 8, heteropolar double; Fig. 9, multiple monster containing a projection (*a*) with one set of structures and another projection (*b*) with two sets of structures lying parallel and having the same polarity; Fig. 10, small multiple monster; Fig. 12, homopolar double; Fig. 13, homopolar double undergoing fission; Fig. 14, homopolar doubles produced by fission of a homopolar double; Fig. 15, "pseudo-conjugant" normal singles united laterally except at anterior end; Fig. 16, "pseudo-conjugant" normal singles united laterally in mid-region.

vidual with normal structure. Rarely, a projection (Fig. 9*b*) contained two sets of structures with the same polarity; fission across such a projection would yield a homopolar double.

Homopolar doubles are of particular importance, from the point of view of the present paper, because they gave rise by fissions to biotypes

of homopolar doubles, as shown in Figs. 13 and 14. The production of multiple monsters from chains and the formation of homopolar doubles by multiple monsters occurred not only once, but many times. All the homopolar doubles studied gave rise to biotypes of doubles like themselves.

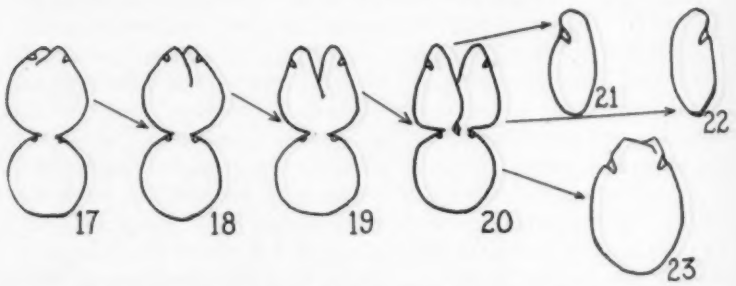
Similar doubles were formed once in an entirely different way. This occurred in a culture of singles left in a Columbia dish a day after most of the colpidia had been removed from it to a finger bowl. (This culture had been fed rye infusion inoculated with *Micrococcus*.) Two individuals (Fig. 15) were found loosely united laterally except at the anterior ends where they did not touch each other; two other individuals were loosely united laterally in the mid-region, but were free at both ends (Fig. 16). These pairs were isolated. Next day, the former had divided into two completely fused pairs; the latter into one completely and one incompletely fused pair. The descendants of each completely fused pair remained united and, like the homopolar doubles produced by multiple monsters, gave rise to biotypes of doubles. These biotypes were kept for three months and, as far as could be judged, could have been kept indefinitely. Thus, in addition to their origin from multiple monsters, biotypes of homopolar doubles arose by lateral fusion of two singles. The superficial resemblance of this fusion to conjugation comes to mind at once, but there are two things that should be remembered in this connection. In the first place, no other phenomenon which might be called normal or abnormal conjugation was ever observed during four years of observation of this species. Secondly, fusions resulting in permanent doubleness are not to be observed in true conjugation. Biotypes of doubles formed by this "pseudo-conjugation" were maintained in mass cultures but not studied farther. In all that follows, it is to be understood that the biotypes of doubles referred to are those that arose from multiple monsters.

V. The Permanence and Stability of Doubles

In both mass and isolation cultures, in both *Achromobacter* and *Micrococcus* fluid, some doubles divided to form two singles and one double instead of two doubles. This came about in the following way. Some doubles (Fig. 17) possessed a small median cleft in the anterior end. In the anterior product of division (Fig. 18) of such an animal, the cleft was usually deeper. Successive anterior products of division (Figs. 19 and 20) contained deeper and deeper clefts until finally one animal (Fig. 20) contained so deep a cleft that the usual transverse fission resulted in the production of two singles (Figs. 21 and 22) from the anterior end, one double (Fig. 23) from the posterior end. The singles and double thus formed reproduced true to their respective types.

This production of singles by doubles raises the question of the permanence of doubles. Will doubles all eventually transform into singles? Or can doubles be maintained indefinitely? As will appear, the answer to these questions depends somewhat on whether mass cultures or isolation cultures are studied. The details of this matter in the two types of cultures will now be set forth.

On April 12, 1930, a mass culture was started of each of eight clones [4] of doubles. These were cultivated in rye infusion inoculated with *Achromobacter,* but the cultures were not kept strictly pure in bacterial content. To each culture fresh fluid was added daily for three or four days, until the original individuals had multiplied to the capacity of the small Columbia dishes. Then the fluid was vigorously sucked up



FIGS. 17–23. Origin of singles from homopolar doubles. Figs. 17, 21, 22, and 23 from camera lucida drawings; Figs. 18, 19, and 20 from notebook sketches. Fig. 17, homopolar double with short median anterior cleft. Fig. 18, double derived from the anterior part of the double shown in Fig. 17; the cleft is deeper. Fig. 19, double derived from anterior part of double shown in Fig. 18; the cleft is still deeper. Fig. 20, double derived from the anterior part of the double shown in Fig. 19; the cleft extends posteriad beyond the fission plane. Figs. 21 and 22, singles derived from anterior part of double shown in Fig. 20. Fig. 23, double derived from posterior part of double shown in Fig. 20.

and expelled from a pipette until the animals were uniformly distributed throughout the fluid and a sample of one or two drops was used to start a new culture in fresh culture fluid. At intervals of three or four days the process of renewal was repeated and observations were made on the proportions of doubles and singles present. At the end of 41 days (May 22), after twelve renewals of the cultures, all clones of doubles except one (clone 2) had transformed into populations consisting exclusively of singles.

[4] The word "clone" is used here in the usual sense of one individual and its vegetative descendants. Arbitrarily, each double produced by a multiple monster has been considered as giving rise to a different clone; this is meant to imply no assumption as to diversity of biotype,—a question treated extensively in Section VI.

A new set of cultures of doubles of these eight clones was started on May 27. In the cultivation of these, the rye infusion was inoculated with *Micrococcus* instead of *Achromobacter*. Renewals of the cultures were made on May 31, June 4, and June 9, before they had to be abandoned. By this time, nine of the cultures had transformed into singles: four of them contained no singles at all, but in the other four 12 per cent to 40 per cent of the colpidia were singles. The average for these four cultures was 26.25 per cent. Comparison with the cultures fed *Achromobacter* after three renewals showed that there were much smaller proportions of singles in the cultures fed *Micrococcus*. Four clones that contained an average of 32.5 per cent singles when fed *Achromobacter* contained no singles when fed *Micrococcus*. The other four cultures averaged 58.75 per cent singles in *Achromobacter*, but only 26.25 per cent singles in *Micrococcus*. This difference in rate of transformation when fed different kinds of bacteria is probably due to a corresponding difference in the rate of multiplication; the colpidia multiplied more rapidly when fed *Achromobacter* than when fed *Micrococcus* (see Table I).

With both types of food, doubles persisted for many generations; hence maintenance of doubleness does not depend on a diet consisting chiefly of *Micrococcus*. However, in both types of food the cultures of doubles gradually contained a larger and larger proportion of singles. In cultures fed *Achromobacter*, all clones (except clone 2) eventually contained only singles. Had the cultures fed *Micrococcus* been observed longer, in all probability they too would have become completely transformed, though this doubtless would have required a longer time than in the cultures fed *Achromobacter*.

It is important to know how this change from doubles to singles took place. Was it a consequence of the death of all doubles or of the transformation of them all into singles? Or was it a consequence of the elimination of doubles in some other way? If the former alternative, then the biotypes of doubles are essentially "Dauermodificationen"; if the latter, they may not be transient biotypes at all. That the latter alternative is the correct one will appear from the records of isolation cultures given below. Meanwhile it is of interest to discover what factors were responsible for the elimination of doubles from mass cultures.

Obviously, an essential factor in this transformation was the production of singles by doubles. If this occurred repeatedly and was not counterbalanced by other factors, it alone would eventually result in the replacement of doubles by singles under the conditions of culti-

TABLE II

Comparison of fission rates of doubles and singles derived from them. Fission rates given in mean number of divisions per line per day.

	PERIOD	June 4–11	June 17–21	June 27–July 1	July 4–7	July 8–11	July 12–16	WEIGHTED MEAN
Clone 3	Number of singles	5	9	11	48	42	39	—
	Means for singles	2.80	2.73	3.11	2.81	3.61	3.59	3.240
	Means for doubles	2.79	2.74	2.90	2.72	3.31	3.33	3.063
	Number of doubles	6	10	12	47	44	45	—
Clone 7	Number of singles	4	—	2	42	39	33	—
	Means for singles	2.69	—	3.00	2.76	3.65	3.41	3.224
	Means for doubles	2.52	—	2.64	2.48	3.11	3.12	2.851
	Number of doubles	6	—	9	46	40	39	—

vation here employed. The doubles fail to double their number at the fissions which yield one double and two singles; but the singles double their number at every fission and the proportion present is continually being increased by the transformation of some doubles into singles. Hence, in the sampling method of culture renewal here employed, eventually too small a proportion of doubles will be present to find a place in the sample. Thus the rate of transformation of a series of cultures would depend on the frequency with which doubles produce singles. This frequency was not ascertained, so that the relative importance of this factor remains unknown.

Other factors, however, become important as soon as some singles have been produced. The subsequent changes in proportion of the two types present must then depend partly on their relative rates of multiplication and partly on their relative rates of mortality.

The fission rates of the singles and doubles of clones 3 and 7 are given in Table II. In clone 3, the singles reproduced 0.1 to 0.3 fissions per day more rapidly than the doubles in all periods except the first two. In these two periods there was practically no difference (0.01 fission per day) between the means for the two groups. However, there were fewer (ten or less) lines in each group during these periods than in any of the later ones; hence, the comparisons during these periods are correspondingly less valuable than during the other periods in which the difference between the two groups was well marked. In clone 7, there can be no doubt that the singles reproduced more rapidly than the doubles. The difference is well marked in all periods for which comparisons are available, even in those in which but few lines were compared. It can be said, therefore, that certainly in clone 7, and very probably in clone 3, the singles reproduced more rapidly than the doubles.

The mortality rates for these groups are given in Table III. With a negligible exception in clone 3, during the period July 4–7, the mortality of singles in both clones 3 and 7 is consistently greater than the mortality of the corresponding doubles. In both clones the mortality rate for the total of all periods is over twice as great among the singles as among the corresponding doubles.

Therefore, in the series of mass cultures of doubles, the gradual change of the proportions of doubles and singles present was not brought about by differential mortality,—indeed was retarded by it; but was due to the continual production of singles by doubles and to the faster reproduction of the singles thus produced. That the death of all doubles or the transformation of them all into singles played no rôle in their extinction is demonstrated by the following account of isolation cultures of doubles.

On April 22, 1930, 48 lines of doubles were isolated in concavities on culture slides. Among these lines eight clones were represented by six lines each. Each line was cultivated in isolation until June 2, 1930. During these 42 days of culture, every clone continued to maintain itself as doubles; no clone died out or transformed completely into singles.

TABLE III

Comparison of mortality rates of doubles and singles derived from them

		PERIOD	June 17–21	June 27–July 1	July 4–7	July 8–11	July 12–16	TOTALS AND MEANS
Clone 3	Singles	No. line-days	50	60	192	188	230	720
		No. died	0	1	0	6	9	16
		No. deaths per 100 line-days	0.0	1.7	0.0	3.2	3.9	2.2
	Doubles	No. deaths per 100 line-days	0.0	0.0	0.05	2.1	0.9	1.0
		No. died	0	0	1	4	2	7
		No. line-days	50	60	191	190	234	725
Clone 7	Singles	No. line-days	—	52	186	179	218	635
		No. died	—	9	8	8	16	41
		No. deaths per 100 line-days	—	17.3	4.3	4.5	7.3	6.5
	Doubles	No. deaths per 100 line-days	—	3.3	1.1	4.4	3.1	2.9
		No. died	—	2	2	8	7	19
		No. line-days	—	60	188	183	225	656

Singles appeared occasionally along with the doubles, especially during the early history of the lines; but during the last 18 days of cultivation no singles were produced in any of the lines. This change in the frequency with which singles were produced was probably a consequence of the method of selection followed each day, for each day the line was perpetuated by the most perfectly doubled individual present (that is,

by the one showing the least development of an anterior cleft). The period (42 days) during which these isolation cultures were maintained as doubles was as long as the period (41 days) during which the mass cultures had transformed completely to singles. Further, these same clones of doubles were maintained from April 5 until October 15. Part of this time they were in isolation culture, part of the time in mass culture; in the latter renewals were made by selecting chiefly doubles. As the usual rate of reproduction was three fissions per day, about 582 generations must have passed while the colpidia remained double. At the end of this time the cultures were discontinued, but there was no reason to suppose that the doubles could not have been maintained indefinitely.

The stability of the doubles was further demonstrated by their maintenance of organization through encystment. The one time encystment was observed in four years of close attention to *Colpidium campylum*, it

FIGS. 24 and 25. Both from notebook sketches and measurements made with ocular micrometer. Fig. 24, cyst 164 μ x 85 μ containing three double colpidia, two small and one large. Fig. 25, one of these doubles immediately after excystment.

occurred in a line of double animals, during isolation culture, 34 days (more than 100 generations) after this line originated from a multiple monster. The cyst (Fig. 24) was discovered less than 24 hours after it formed; in it there were three vigorously moving doubles. Of these three, one was large, two small. Within half an hour, the larger animal divided into two. The cyst remained in this condition one more day. On the third day, five animals were present in the cyst; on the fourth day, six animals. On the fifth day, the cyst was opened with a fine glass needle to allow the encysted animals (of which one is shown in Fig. 25) to emerge. Each of these was a double animal; four of them were used to initiate separate lines of descent. Records showed no differences among the emerged animals and their descendants or between the descendants of encysted and non-encysted members of the same clone, so that more detailed studies on this matter were not made. However, the important point is this: in spite of the reorganizations known to occur in cysts of ciliates, the encysted colpidium did not reorganize as a normal single, but emerged from as it had entered the cyst,—with the double organization.

VI. Diversity of Biotype Among Doubles and Among the Singles Produced by Them

The origin of diverse biotypes during vegetative reproduction has been the subject of numerous investigations (see review by Jennings, 1929). To these must be added the present one on *Colpidium*, in which it has been shown (Section IV) that biotypes of doubles originate from a clone of singles under the influence of a special environmental condition (diet including *Micrococcus*). Further (see Section V), some individuals in these biotypes of doubles give rise to new biotypes of singles. We take up in this section the question of whether there arise other biotypic diversities among the doubles and the singles produced by them. Of this question there are several aspects: (1) Do biotypic diversities exist among the different clones of doubles? (2) Do

Table IV

Comparison of fission rates of doubles of clones 3 and 7. Rates given in number of divisions per line per day.

PERIOD		May 1–7	May 8–14	June 4–11	June 17–21	June 27–July 1	July 4–7	July 8–11	July 12–16	Weighted mean for 8 periods
Clone 3	No. lines	6	6	6	10	12	47	44	45	
	Fission rate	3.05	3.05	2.79	2.74	2.90	2.72	3.31	3.33	3.064
Clone 7	Fission rate	2.95	2.73	2.52	2.58	2.64	2.48	3.11	3.12	2.835
	No. lines	6	6	6	10	9	46	40	39	
Clone 3 minus Clone 7		0.10	0.32	0.27	0.16	0.26	0.24	0.20	0.21	0.229

biotypic diversities exist among singles derived from different clones of doubles? (3) Do biotypic diversities exist among singles produced independently from the same clone of doubles? (4) Do biotypic diversities exist between singles produced by doubles and singles not descended from doubles? (5) Do biotypic diversities exist among different lines of descent within a clone? These questions will now be taken up in the order mentioned.

(1) *Biotypic diversities among different clones of doubles.*—Eight clones of doubles were compared in (*a*) the rate of decrease in proportion of doubles present in series of mass cultures; (*b*) the rate of multiplication; and (*c*) the rate of mortality.

(*a*) Striking differences in the rate of decrease in proportion of doubles present appeared among the clones cultivated in series of mass cultures, as described in Section V. In the series fed *Achromobacter,* at the end of the period of observation, no doubles remained in any of the eight clones, except in clone 2. In this clone, however, the vast majority of colpidia present were still doubles. Clearly, the rate of decrease in proportion of doubles was less in clone 2 than in any other clone. This was apparent in both types of food cultures and at all stages in the series. For example, after three renewals of the cultures, all colpidia of clone 2 were still double when fed *Micrococcus* and 95 per cent were double when fed *Achromobacter*. But in clone 3 only 60 per cent of the colpidia were double in *Micrococcus* and only 5 per cent in *Achromobacter*. Between these two extremes, in clone 8, 88 per cent of the colpidia were doubles in *Micrococcus,* 60 per cent in *Achromobacter;* and in clone 1, 72 per cent were doubles in *Micrococcus,* 40 per cent in *Achromobacter*. The different clones thus manifested at least four different rates of transformation and these differences between the clones were the same in both types of culture fluid.

(*b*) An extensive comparison was made of the rates of multiplication in clones 3 and 7. Their mean fission rates for eight periods of from four to seven days each are given in Table IV. In all periods clone 3 multiplied more rapidly than clone 7. At the end of the third period, the slowest line of clone 3 was selected to give rise to all later members of the clone; at the same time, the fastest line in clone 7 was selected to give rise to all later members of this clone. As appears in the table, this radical adverse selection in both clones changed neither the direction nor the magnitude of the difference between the two clones. Clone 3, for the eight periods, had a mean rate of 3.064 fissions per line per day; during the same time, clone 7 had a mean rate of 2.835 fissions per line per day. The different periods gave results very similar to the general mean: in five of the eight periods the excess of clone 3 over clone 7 was between 0.20 and 0.27 fission per line per day; in two periods it was below this range (0.10 and 0.16 fission) and in one period above it (0.32 fission). There can be no doubt of the uniform hereditary difference between clones 3 and 7 in fission rate.

(*c*) The rates of mortality were also extensively compared in these two clones (see Table V). In seven periods, extending over a period of 86 days and including records for 54 of these days, the mortality rates were 0.78 deaths per 100 line-days in clone 3 and 2.40 deaths per 100 line-days in clone 7. In no period is the mortality rate of clone 3 higher than that of clone 7. The difference in mortality rates of these

two clones appears constantly through all parts of the experiment and is thus a biotypic diversity.

Among the clones of doubles here compared, clones 2 and 8 arose at different times from one multiple monster and the other six clones arose at different times from another multiple monster. Thus, differences in the rate of decrease in proportion of doubles present in series of mass cultures existed between clones derived from different multiple monsters: clones 2 and 8 as compared with clones 1 and 3; but similar differences also existed between clones derived from the same multiple monster: the rate in clone 2 differing from that in clone 8, and the rate in

TABLE V

Comparison of mortality rates of doubles of clones 3 and 7

	PERIOD	April 22–May 14	June 4–11	June 17–21	June 27–July 1	July 4–7	July 8–11	July 12–16	TOTALS AND MEANS
Clone 3	No. line-days	120	48	50	60	191	190	234	893
	No. died	0	0	0	0	1	4	2	7
	No. deaths per 100 line-days	0.0	0.0	0.0	0.0	0.05	2.1	0.9	0.78
Clone 7	No. deaths per 100 line-days	1.7	0.0	0.0	3.3	1.10	4.4	3.1	2.40
	No. deaths	2	0	0	2	2	8	7	21
	No. line-days	120	48	50	60	188	183	225	874

clone 1 differing from that in clone 3. Likewise, differences between clones of doubles (clones 3 and 7) derived from the same multiple monster were found in rate of fission and in rate of mortality.

(2) *Biotypic diversities among singles derived from different clones of doubles.*—Clones 3 and 7 of doubles have just been shown to differ in rate of fission and in rate of mortality. Do the singles produced by these two clones of doubles differ in the same way? One single from each of these two clones of doubles was permitted to give rise to a number of lines and the mean fission rates of these two groups of singles were compared in four periods. The differences found were small and not constant, so that no significance may be attached to them. In mortality rate, however, the situation was different. As appears in Table VI, in all four periods the rate of mortality is greater—usually very

much greater—among the singles of clone 7 than among the singles of clone 3. The rate for the total time is 2.39 deaths per 100 line-days in clone 3 and 6.33 deaths per 100 line-days in clone 7. The rate for clone 7 is thus 2.65 times as great as that for clone 3. In connection with this difference, it is of interest to note (see Table V) that the doubles of clone 7 had a mortality rate 3.08 times as great as the doubles of clone 3.

TABLE VI

Comparison of mortality rates of stocks of singles produced by one single from a clone 3 double and one single from a clone 7 double

	PERIOD	June 4–11	June 27–July 1	July 4–7	July 8–11	July 12–16	TOTAL
Singles from clone 3 double	No. of line-days	42	60	192	188	230	712
	No. of deaths	1	1	0	6	9	17
	No. of deaths per 100 line-days	2.4	1.7	0	3.2	3.9	2.39
Singles from clone 7 double	No. of line-days	44	52	186	179	218	679
	No. of deaths	2	9	8	8	16	43
	No. of deaths per 100 line-days	4.5	17.3	4.3	4.5	7.3	6.33
Rate of clone 7 singles minus rate of clone 3 singles		2.1	15.6	4.3	1.3	3.4	3.94

(3) *Biotypic diversities among singles produced independently from the same clone of doubles.*—As set forth above, singles were produced two at a time from the anterior half of a double animal. One arises on the right side, one on the left (see Fig. 20). Do the two singles of such a pair differ? That there might be a difference in symmetry was suggested to me by Mr. Donald Costello; but I was unable to detect it. However, it was clear that the two singles of a pair differed sometimes, but by no means always, in other respects. These differences occurred when the anterior cleft of the parental double was much shifted from the usual median position. Frequently the narrower part was separated off as a single one division or more before the broader part. The single produced from the narrower part was invariably narrow, pale, and short as compared with the single produced from the broader part or with the singles ordinarily produced from doubles. The fates

of these two different types of singles were frequently observed. The difference in their fates is illustrated by an experiment on 48 lines observed from May 16–19, 1930. In this group, of the seven singles derived from the narrower parts of unequally cleft doubles, the progeny of four died. Among the 41 lines not derived from narrow parts of unequally cleft doubles, the progeny of only two died. Thus 57 per cent of the one group died as compared with only 5 per cent of the other group. Many other observations confirmed the results in these groups, so that there was no doubt of the very much greater mortality among

TABLE VII

Comparison of mortality rates of a group of singles descended from one single produced by a double of clone 7 and one single not descended from doubles.

	PERIOD	June 27–July 1	July 4–7	July 8–11	July 12–16	TOTAL
Singles not descended from doubles	Number of line-days	479	384	376	473	1712
	Number of deaths	5	3	10	8	26
	Number of deaths per 100 line-days	1.0	0.8	2.7	1.7	1.52
Singles from clone 7 double	Number of line-days	52	186	179	218	635
	Number of deaths	9	8	8	16	41
	Number of deaths per 100 line-days	17.3	4.3	4.5	7.3	6.46
Singles of clone 7 minus singles not descended from doubles		16.3	3.5	1.8	5.6	4.94

the descendants of singles derived from the narrower parts of unequally cleft doubles. Whether the singles produced from the ordinary medially cleft doubles at one time were diverse from those produced from a double of the same clone at another time was not investigated.

(4) *Biotypic diversities between singles descended from doubles and singles not descended from doubles.*—Comparisons were made between singles descended from doubles and singles not descended from doubles in rate of fission and in rate of mortality. The differences found in rate of fission were small and not consistent, so that no significance may be attached to them. In rate of mortality, however, the differences were clear (see Table VII). The total difference between the singles not de-

scended from doubles and a group of singles descended from one single produced by a double of clone 7 is very great: the mortality rate of the latter group is 4.25 times as great as that of the former. This difference is clearly manifested in every period and demonstrates a biotypic difference in rate of mortality between these two groups of singles.

(5) *Biotypic diversities within a clone.*—As already set forth, the most striking differentiation into biotypes that occurs within a clone of doubles is its splitting into biotypes of singles and doubles. This occurred repeatedly in all clones of doubles studied. Furthermore, the biotypes of singles so formed within a clone of doubles were not all alike: some were normal singles, others were narrow, pale, and highly inviable. In addition to these biotypic differences, there were indications of other biotypic differences within a clone, among the doubles themselves. In the isolation cultures of doubles maintained from April 22 until June 2, the frequency with which singles were produced changed strikingly. This was probably brought to light by the practice of selecting daily from among the individuals produced during the previous 24 hours in each line, the individual which showed least development of an anterior cleft. In each line this individual was used to perpetuate the line and the remaining individuals were discarded. In spite of this method of selection, cleft individuals continued to appear and give rise to singles during the early history of these isolation lines. During April 22–30, singles were produced in 31 of the 48 lines of doubles under cultivation. In some lines they appeared more than once. The product of the number of lines in which they appeared by the number of days on which they appeared gives a measure of their frequency of production. This product was 63 line-days, yielding an interval of 6.86 line-days between successive productions of singles. These figures are in striking contrast to those obtained for the same lines during the period May 16–June 2. Not one single was produced during these 864 line-days. In order to bring to light such a great change in the frequency with which doubles produced singles within the same lines of descent, there would have to be genetic differences in frequency of single production among the doubles of each clone. Such a conclusion seems required by the evidence.

Attempts to isolate by selection biotypic differences in rate of fission within clones of doubles, within clones of singles, and within clones of singles descended from doubles were all fruitless. The coefficient of variation of fission rate of a clone of doubles (8.38 per cent) was greater than the corresponding coefficients for singles descended from this clone of doubles (6.46 per cent) and for a clone of singles not descended

from doubles (6.66 per cent). But this greater variability of the clone of doubles was probably not an index of the existence of biotypic diversities in fission rate within this clone, because extreme selection did not result in the isolation of sub-clones with diverse fission rates.

VII. Discussion

The racial effects of environmental conditions found here in *Colpidium* bear a striking parallel in many respects to the relations previously found in the rhabdocoel turbellarian, *Stenostomum incaudatum* (Sonneborn, 1930*b*). As in *Colpidium*, so in *Stenostomum*, special environmental conditions induced abnormalities in reproduction resulting in the formation of irregular monsters. These, likewise, gave rise to double animals that differed from each other and produced races of doubles differing in the same way. Further, in both *Colpidium* and *Stenostomum* the races of doubles maintained their character after removal from the environment that induced their formation, except that singles were formed whenever a cleft of sufficient extent occurred in the growing region perpendicular to the plane of fission. In both, singles gave rise to races of singles of higher viability than the doubles from which they arose.

The degree of similarity between a protozoan and a flatworm in the effects of environmental conditions on their hereditary characteristics is particularly striking in contrast to the very different results of most similar work on higher organisms. What is the basis of this difference? It seems to be the method of reproduction. In sexual reproduction, change of hereditary characters depends largely on changes in the nature or in the composition of the chromatin. Environmental conditions of special penetrability are required to get at this material, so that but few environmental conditions are effective in altering hereditary characteristics. In asexual reproduction, on the other hand, change of hereditary characters may be brought about without in the least affecting the nature or composition of the chromatin; changes in the composition of components of a larger order are also capable of self-perpetuation—that is, are heritable. Examples of this are the rearrangement of parts in homopolar doubles in *Stenostomum* and *Colpidium*. That changes in the chromatin were not involved in these examples was demonstrated by the fact that when individuals were produced from cleft parts of doubles, these were always singles and gave rise to biotypes of singles. The type of hereditary change involved in the production of biotypes of doubles in *Stenostomum* and *Colpidium* is similar to the type involved in the production of stocks of *Drosophila* in which the two X-chromo-

somes are united or in which translocations, inversions, or reduplications have occurred. All such examples illustrate hereditary changes not due to changes in the nature of the germinal material, but due to changes in the number of units or arrangement of units in the germinal material.

It is remarkable that very diverse environmental conditions acting on such diverse organisms as *Colpidium* and *Stenostomum* should result in similar stable types. It may be that this is another example of the stability of whole multiples, as in polyploids; and that of all the teratological consequences of diverse original stimuli, the whole multiples that result are particularly of the viability requisite for survival and self-perpetuation.

VIII. Summary

In a clone of *Colpidium campylum* (Stokes), a small proportion (less than 1.2 per cent) of the individuals formed chains when cultivated in a rye infusion inoculated with the bacterium *Micrococcus sp.* (probably *sensibilis*), but not when the infusion was inoculated with *Achromobacter sp.* (probably *candicans*). Other factors, one of which possibly was the concentration of colpidia in the culture fluid, affected the proportion of chains formed when the colpidia were fed *Micrococcus*. Chains thus produced went through a series of developments including the formation of heteropolar doubles and multiple monsters, and culminating often in the formation of homopolar doubles of a self-perpetuating, relatively stable sort. Similar biotypes of doubles were also formed once as a result of a "pseudo-conjugation." In clones of homopolar doubles, singles sometimes arose by ordinary transverse fission across a double with a deep median anterior cleft. Consequently, mass cultures begun with doubles eventually contained singles also. As the two types multiplied side by side, the relative proportion of singles gradually increased. When the cultures were regularly renewed by taking a sample of the old culture to start a new one, eventually, after many such renewals, doubles entirely disappeared from the cultures, leaving only singles. The change occurred in both *Achromobacter* and *Micrococcus* fluid, but more rapidly in the former; this was probably due to the more rapid reproduction in that fluid. The change in the mass cultures was not due to dying out of doubles or to the transformation of all of them into singles. In isolation culture, lines of doubles were maintained as long as the period required for doubles to disappear entirely from mass cultures. Furthermore, when doubles were deliberately salvaged at each renewal of culture, they were maintained in cultivation, partly in isolation, partly in mass, for 194 days, during which about 582 generations passed. The disappearance of doubles

from mass cultures in 41 days or less must therefore have been due to other factors than the inability of doubles to live and reproduce their kind. One of these factors was a differential rate of fission: the singles produced by the doubles of one clone multiplied 0.373 fissions per line per day more than the doubles of this clone. On the other hand, differential mortality counteracted this to some extent, for the mortality rate of singles was higher than the mortality rate of the doubles that produced them. The gradual disappearance of doubles in series of mass cultures was therefore due partly to their repeated production of singles and partly to their lower fission rate. The persistence of doubles during nearly 600 generations, by the end of which time no evidence of inability to maintain themselves had yet appeared, indicates that the type could have maintained itself indefinitely, even when the bacterium that led to its formation was absent or present in but very small quantities. Further evidence of stability of organization was the passage of a line of doubles through encystment without loss of the double organization.

The question of whether diverse biotypes existed among the experimentally produced doubles and their descendants was extensively investigated. (1) Different clones of doubles differed (*a*) in the rate at which doubles disappeared from series of mass cultures, (*b*) in rate of multiplication, (*c*) in rate of mortality. (2) Singles derived from diverse clones of doubles differed in rate of mortality to about the same extent as the clones of doubles from which they had been derived. (3) There were two very different kinds of singles derived from the same clone of doubles: the usual kind and a rarer kind formed by transverse fission across an unequally cleft double. The singles formed from the narrower anterior part of these were narrower, paler, and shorter than ordinary singles and had a much higher rate of mortality. (4) Singles not descended from doubles had a lower rate of mortality than singles descended from doubles. (5) Within a clone of doubles there were genetic differences in the frequency with which singles were produced, for long-continued selection within lines of doubles brought to light very great changes in this frequency. Attempts to isolate by selection biotypic diversities in rate of fission within clones of doubles and of singles failed, although the coefficient of variation of fission rate was higher for doubles than for singles.

The general picture of the genetic consequences of environmental action in the ciliate protozoan, *Colpidium campylum*, is strikingly similar to the picture in the rhabdocoel turbellarian, *Stenostomum incaudatum*. The similarity in these and the difference of both from the genetic effects of environmental action in higher organisms were ascribed to the method of reproduction. In asexual reproduction hereditary

changes may arise without altering the nature of the chromatin; they may be due simply to changes in the number of units or arrangement of units in the self-perpetuating parts. The changes induced in *Colpidium* and *Stenostomum* were of this sort and, in this respect, resemble translocations, inversions, and reduplications in *Drosophila*.

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CHROMOSOMES OF ARTIFICIALLY ACTIVATED EGGS OF URECHIS

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The eggs of *Urechis* that cleave and develop as a result of activation by dilute sea water have been previously shown (Tyler, 1931*a*) to be those which extrude no polar bodies. It would appear then that the embryos produced by such eggs might be tetraploid, diploid, or haploid, depending upon the behaviour of the chromosomes during the first two nuclear divisions. A cytological investigation of such eggs shows that the embryos are diploid in chromosome number, and that only one maturation division occurs.

The preparations were made by a method used by Karl Bělař and similar to that described by him (1928). It consists of joining and later separating two cover-slips, one containing a drop of eggs and the other a drop of fixing fluid. The eggs are flattened to any desired extent and stick to the cover-slips, which can be handled in the same manner as slides containing sectioned material.

Two types of eggs are produced as a result of activation with dilute sea water (Tyler, 1931*a*). In one type the initial behaviour is identical with that of the normally fertilized eggs, two polar bodies are produced but none of the eggs divide. In the other type the initial behaviour is quite different from that of normally fertilized eggs; no polar bodies are produced but practically all the eggs of this type divide and develop.

In making the cytological preparations of the eggs of the first type, use was made of the fact, previously reported (Tyler, 1931*b*), that an inverse relation exists between the total percentage of activation and the percentage of cleavage. Thus treatments giving 100 per cent activation produce only eggs of the first type which do not divide. For preparations of the type which does not extrude polar bodies, the eggs had to be isolated from dishes containing also unactivated eggs and activated eggs of the first type. These eggs can be readily distinguished at an early stage and can be removed for cytological preparations before the time at which the first polar body appears in the eggs of the first type.

The counts of chromosome number were generally made from polar views of anaphase groups inasmuch as precociously divided chromosomes in metaphase might cause difficulty.

The Eggs That Produce Polar Bodies

The behaviour of the chromosomes in the maturation division of the artificially activated eggs that extrude two polar bodies is identical with that of the normally fertilized eggs. The normal diploid number in *Urechis* is most probably thirty-six chromosomes and the haploid number eighteen. The variability in the chromosome numbers shown in the tables is undoubtedly due to errors in counting. The artificially activated eggs (last section of Table I) show the haploid number of

Table I

FIRST CLEAVAGE OF NORMAL FERTILIZED EGGS		FIRST POLAR DIVISION OF NORMAL FERTILIZED EGGS		FIRST POLAR DIVISION OF PARTHENOGENETIC EGGS	
Chromosome Number	Number of Groups	Chromosome Number	Number of Groups	Chromosome Number	Number of Groups
33	4	15	3	14	2
34	6	16	2	16	5
35	3	17	8	17	10
36	7	18	8	18	6
37	2	19	1	19	1

chromosomes at the first maturation division. The second polar division also occurs normally and the egg is left with the haploid number of chromosomes, which form a nucleus and move into the center of the egg. A large monaster then forms at about fifteen minutes after the extrusion of the second polar body and the chromosomes distribute themselves irregularly about the astral rays. The monaster disappears and a vesicular nucleus is formed about ten minutes later. The monaster then reappears about twenty minutes later and a larger and variable number of chromosomes are seen. The monaster may disappear and reappear a third time. This behaviour is essentially similar to that described by Herlant (1918) in the sea-urchin for eggs activated by butyric acid. The failure of the eggs of this type to divide appears then to be due to the failure to form an amphiaster.

The Eggs That Divide

In the eggs of this type the germinal vesicle breaks down and tetrad chromosomes appear at about twenty to twenty-five minutes after treatment. At this time the first polar spindle appears in the control eggs. But no spindle is seen in the eggs of this type and at about ten to twenty minutes later the tetrads each form a small vesicular karyomere. The

nucleolus generally persists as such throughout this time and about seventeen or eighteen karyomeres may be seen distributed throughout the egg. Later a single large nucleus is formed apparently by the fusion of the karyomeres. The nucleolus remains intact and is seen within the nucleus. This nucleus is generally about two-thirds of the size of the original germinal vesicle and has a granular appearance similar to that of the cytoplasm. The eggs remain in this condition for about an hour, after which the first cleavage spindle appears.

TABLE II

Parthenogenetic Eggs

FIRST CLEAVAGE		SECOND CLEAVAGE		THIRD CLEAVAGE	
Chromosome Number	Number of Groups	Chromosome Number	Number of Groups	Chromosome Number	Number of Groups
13	1	32	2	32	5
15	4	33	2	33	7
16	8	34	3	34	6
17	5	35	8	35	4
18	5	36	4	36	5
		38	1	37	2
				38	2
				40	1
		45	1	13	2
		46	1	14	2
		48	2	15	2
		50	1	16	4
		53	2	18	5
		54	1	20	1

An attempt was made to determine whether any division of the chromosomes occurred prior to the first cleavage, and whether fusion of the egg nucleus with a submerged polar body nucleus such as described by Morris (1917) in *Cumingia,* occurred in *Urechis.* The evidence shows that such behaviour does not occur in *Urechis.* It is possible to determine this point with some certainty in *Urechis* inasmuch as the eggs which are to divide remain indented until just before the first cleavage. The indented eggs were therefore preserved at close intervals up to that time. No division figures or fusion of two nuclei were observed. Furthermore, the chromosomes on the first cleavage spindle have the appearance of tetrads and are eighteen in number. In anaphase they open out as typical dyads.

It appears then that the first cleavage spindle is identical with the first polar spindle as far as the chromosomes are concerned, and the first division may be considered a maturation division.

At the second cleavage the diploid number of chromosomes is usually seen. This is the case with the twenty anaphase chromosome groups of the five eggs listed in Table II. The chromosomes of these groups occur in more or less closely associated pairs. This means that the chromosomes had divided previous to this division. In other words the second cleavage is a mitotic rather than maturation and the diploid number of chromosomes is retained. In two eggs listed in Table II eight anaphase groups gave chromosome counts approximating fifty-four,

TABLE III

Parthenogentic Eggs

FOURTH CLEAVAGE		EMBRYOS	
Chromosome Number	Number of Groups	Chromosome Number	Number of Groups
31	2	28	2
32	6	29	1
33	8	30	4
34	7	32	6
35	6	33	3
36	10	34	7
37	4	35	2
38	3	36	3
39	1	38	2
40	1		

the triploid number. The origin of such chromosome groups was not determined, and they were not encountered in the slides of the later stages.

In the anaphase of the third cleavage the diploid number is again usually seen. Two eggs were obtained, however, in which the chromosomes were of the haploid number. These must have arisen by the occurrence of both maturation divisions in the first two cleavages of the egg. The haploid number was not obtained again in the later stages of other eggs studied.

The chromosome counts at the fourth cleavage of the egg, Table III, again approximated thirty-six, the diploid number. For the embryos, normal top-swimmers were isolated and preserved in the usual manner. Counts on fifteen pairs of anaphase groups (Table III) showed that the diploid number is present.

The failure of the artificially activated egg that extrudes polar bodies to divide may be attributed to its retaining only the haploid number of chromosomes or to the possession of only the inner central body of the second polar spindle which is incapable of forming an amphiaster. The former is an unlikely assumption inasmuch as some haploid cleavage has been obtained in *Urechis*. But if the interpretation is based upon the behaviour of the central bodies,[1] it is difficult to see why the cleavage of those eggs that produce no polar bodies should go beyond the four-cell stage. At this stage the centrosomes should be equivalent to the three that would have gone into the polar bodies and the one that remains in the egg. It might appear then that when the polar body central bodies come to lie within the egg cytoplasm they are capable of forming amphiasters. It may be pointed out in this connection that the first polar body in *Urechis* may or may not divide. Correspondingly in the artificially activated eggs, one of the cells of the two-cell stage often fails to divide. Similarly in the four-cell stage one of the cells often fails to divide corresponding to the egg cell that receives the inner central body of the second polar spindle. This again may be interpreted to mean that the first polar spindle is used for the first division. However, in a large number of cases all four cells divide, and since no accessory asters have been observed in these eggs, it appears that the inner central body of the second polar spindle has regained the ability to form an amphiaster.

Summary

1. The embryos resulting from the artificial activation of *Urechis* eggs are diploid in chromosome number.

2. The diploid number is apparently obtained by the utilization of the first polar spindle for the first cleavage and the substitution of a mitotic division for the second maturation division.

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PRODUCTION OF CLEAVAGE BY SUPPRESSION OF THE POLAR BODIES IN ARTIFICIALLY ACTIVATED EGGS OF URECHIS

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It was suggested in an earlier paper (Tyler, 1931*b*) that those eggs which do not ordinarily divide as a result of artificial activation could be made to do so by suppression of the polar bodies. This was based on the fact that upon activation by means of dilute sea water only those eggs divide that extrude no polar bodies. The other type of egg produced by artificial activation behaves very much like the normally fertilized egg in its initial reactions to the treatment, extrudes two polar bodies, but does not divide. The results of the experiments reported here show that the polar bodies can be suppressed in such eggs by means of a second treatment with dilute sea water and that the eggs then divide.

When *Urechis* eggs are treated with dilute sea water the percentage of the activated eggs that divide after various lengths of exposure bears an inverse relation to the total percentage of activation (Tyler, 1931*b*). Thus exposures resulting in 100 per cent activation give no cleavage, and the eggs are all of the type that extrudes both polar bodies. This simplifies the task of re-treating such eggs, since no unactivated eggs and no eggs of the type that divides are present in the dishes. The percentage of activation can be determined at about ten minutes after the initial treatment, and since the first polar body appears at thirty minutes at room temperature there is ample time for the second treatment.

The first attempts at polar body suppression were made by means of anesthetics such as ether, phenyl urethane, and chloretone in various concentrations. Low temperature was later tried, as was also hypertonic sea water. These agents gave variable results, and in general although the polar bodies were suppressed while the eggs remained under treatment they often appeared later when the eggs were removed to normal sea water.

Dilute sea water was then tried and this was found to be quite an effective agent for suppressing the polar bodies and producing cleavage.

The concentrations used were 50 and 55 per cent sea water. Higher concentrations generally failed to suppress the polar bodies and lower concentrations appeared to injure the eggs.

The Second Treatment with Dilute Sea Water

In these experiments the eggs were first treated for various lengths of time with 30 or 40 per cent sea water. The length of exposure resulting in 100 per cent activation is known fairly well from previous experiments, and so treatments ranging about the optimum time were used. The dishes were then examined to determine which actually

Table I

Re-Treatment with 55 per cent sea water. Eggs first treated for 2 minutes with 30 per cent sea water gave 100 per cent activation and all eggs later showed two polar bodies and no cleavage. First polar body out at 30 minutes; second at 40 minutes. *p.b.* = polar body.

Time after first treatment	Length of second treatment	Cleaved			Uncleaved		
		0 p. b.	1 p. b.	2 p. b.	0 p. b.	1 p. b.	2 p. b.
minutes	*minutes*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
25	5	0	0	0	0	0	100
25	10	2	3	0	0	2	89
25	15	20	6	0.2	2	3	69
25	20	36	2	0	34	12	16
25	25	71	2	0	18	9	0
25	30	94	1	0	3	1	1
25	35	96	2	0	1	1	0
25	40	88	0	0.1	7	3	2
40	5 to 40	0	0	0	0	0	100
55	5 to 40	0	0	0	0	0	100

showed 100 per cent activation. Large samples of eggs were then transferred from such dishes at various times after the beginning of the first treatment to 50 or 55 per cent sea water. The eggs were usually exposed to the second treatment for 5 to 40 minutes. They were examined in the dilute sea water and later in normal sea water to determine whether or not the polar bodies were suppressed by the treatment. The usual precautions in regard to the amount of water transferred with the eggs, etc. were taken.

The results of one such series of experiments are given in Table I. The eggs were first treated with 30 per cent sea water and an exposure of two minutes was found to give 100 per cent activation. All of the

eggs in this two-minute dish later showed two polar bodies. The first polar body appeared at 30 minutes and the second at 40 minutes after the first treatment. A sample of eggs was transferred to 55 per cent at 25 minutes after the first treatment; that is, 5 minutes before the first polar body was due to appear, and treated for various lengths of time. About an hour later the percentage of cleavage was determined and also the presence or absence of polar bodies. As shown in the table, when the eggs are exposed for 5 minutes to the second treatment with dilute sea water the polar bodies appear and no cleavage is obtained as in the controls. However, upon longer exposures fewer of the eggs show two polar bodies, and after exposure of 25 minutes or more practically none of the eggs show two polar bodies. At the same time the percentage of cleavage increases from zero to 98 per cent. The great majority of the cleaved eggs have no polar bodies. A small percentage of the divided eggs have one polar body (column 4 in the table) and a very few of the divided eggs show two polar bodies.

When the second treatment is applied at 40 or at 55 minutes after the first treatment (*i.e.*, after extrusion of the second polar body) the results are the same as for the control eggs—none of the eggs divide.

Fourteen series of experiments of the type illustrated by Table I were run and all gave similar results. Cleavage was obtained when the second treatment was applied before the time of extrusion of the first polar body and was continued until after the time of extrusion of the second polar body. When the eggs were given equivalent treatments at any time after the second polar body had appeared no cleavage was obtained. Cleavage was sometimes obtained when the treatment was applied after the extrusion of the first polar body. Eggs were also isolated after the second treatment according to the number of polar bodies they showed, and of 200 eggs examined cleavage was obtained in 90 per cent of the eggs that showed no polar bodies, 15 per cent of the eggs with one polar body, and none of the eggs with two polar bodies.

At the first cleavage the doubly treated eggs divided into two or three cells. Of 400 eggs on which counts were made 65 per cent divided into two cells and 35 per cent into three cells. Cleavage often stopped in the four-cell stage. Large numbers of abnormal top and bottom-swimmers but no normal embryos were obtained from the re-treated eggs.

It is evident then that when the polar bodies are suppressed by means of a second treatment the eggs are then capable of division. Suppression of one polar body appears to be less effective in this regard than suppression of both.

Cytological preparations were made of the doubly treated eggs according to the method previously described (Tyler, 1932) in order to determine the behaviour of the chromosomes and centrosomes. Eggs were removed for preservation directly from the dilute sea water and also after their return to normal sea water. In the eggs preserved within twenty minutes after the application of the second treatment the achromatic figure was generally not visible, and the chromosomes appeared as condensed bodies, similar to their metaphase condition. They formed a single group at the pole of the egg. In eggs removed at later times from the dilute sea water the chromosomes were often found in two groups of about 12 to 18 each although generally they appeared in one group of about 18 scattered about in the polar region. When the eggs were returned to normal sea water at 30 minutes after the second treatment and later preserved, they first showed two chromosome groups which were generally associated with two separate asters. At later stages the eggs showed a single group of chromosomes, presumably due to the fusion of the two separate groups. The asters are usually not visible at this time. At the time of cleavage an amphiaster develops, and the chromosomes are seen distributed irregularly about the spindle. The first cleavage divides the chromosomes irregularly and counts of anaphase groups ranged from 8 to 40, the two groups sometimes containing equal numbers and at other times radically different numbers of chromosomes. Later stages were not followed.

The examination of the cytological preparations shows that when the polar bodies are suppressed, the chromosomes first separate into two groups which later come together and distribute themselves more or less irregularly about the first cleavage spindle.

Discussion

An important question involved in the cleavage of artificially activated eggs concerns the origin of the amphiaster. The parthenogenesis experiments of Herlant (1918), Fry (1925), and others on the echinoderm egg are generally taken to mean that central bodies and asters may arise *de novo* and either combine or divide to form an amphiaster. Although this argues against Boveri's view of the genetic continuity of the central bodies, more evidence has recently been presented in its favor from other sources (Sturdivant, 1931; Wilson and Huettner, 1931; Pollister, 1930; and Johnson, 1931). The parthenogenesis experiments on *Cumingia* (Morris, 1917; Heilbrunn, 1925) and on *Urechis* (Tyler, 1931*a*) show that cleavage is obtained when the eggs fail to extrude polar bodies. The question arises as to whether in such cases

the first cleavage spindle develops *de novo* or whether it is directly continuous with the first polar spindle. Evidence of the similarity of the first cleavage spindle of such eggs and the normal first polar spindle has been previously presented (Tyler, 1932). In the results presented here it was shown that suppression of the polar divisions enables eggs to divide which would not ordinarily do so. The cytological work is insufficient to determine whether when the polar divisions are suppressed the first maturation spindle is converted into the first cleavage spindle. The two separate asters observed when the polar bodies are suppressed may have been derived from the poles of the first maturation spindle or may have arisen *de novo*. The fact that similar treatments applied after polar body extrusion do not produce such effects favors the former view, but in the absence of more detailed cytological evidence the question as to the origin of amphiaster in the doubly treated eggs still remains open.

The double treatment used here obviously differs from Loeb's classical double treatment for sea-urchin eggs. In these experiments the agent used for the second treatment was of the same type as that used for the first; and its effect was to enable eggs to divide by suppressing the polar bodies. Moreover, Just (1922) has clearly shown that in the sea-urchin egg only a single treatment is necessary, whereas for several different agents used for single treatments on *Urechis*, the optimally activated eggs do not divide. Thus hypertonic sea water alone gives similar results to hypotonic sea water. Hypertonic sea water was also tried on optimally activated eggs after the extrusion of the polar bodies, but no cleavage occurred.

Summary

1. The polar bodies can be suppressed in artificially activated eggs of *Urechis* by means of a second treatment with dilute sea water.

2. The treatment must be applied before the time of extrusion of the first polar body and continued until after the time of extrusion of the second.

3. The eggs in which the polar bodies are thus suppressed undergo cleavage whereas ordinarily they would not do so.

4. Similar second treatments applied after the time of the extrusion of the second polar body do not induce cleavage.

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OSMOTIC PROPERTIES OF THE ERYTHROCYTE

V. The Rate of Hemolysis in Hypotonic Solutions of Electrolytes

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I

In an earlier paper in this series (Jacobs, 1932) it has been shown that, on the assumption that the rate of entrance of water into the erythrocyte in accordance with simple osmotic laws is the factor of chief importance in determining the rate of osmotic hemolysis, the theoretical relation between the time at which some given degree of hemolysis is attained and the osmolar concentration of the surrounding solution ought to be given by the equation:

$$t_h = \frac{1}{k} \cdot \frac{V_0}{A}\left(\frac{c_0}{C^2}\ln\frac{c_0 - C}{c_0 - CR} - \frac{R-1}{C}\right) \tag{1}$$

or, if the external medium be water alone, by

$$t_h = \frac{1}{k}\frac{V_0}{2c_0A}(R^2 - 1), \tag{2}$$

where c_0 is the osmolar concentration of the solution in osmotic equilibrium with the normal erythrocyte, R the ratio of this concentration to that which will just cause the given degree of hemolysis, C the osmolar concentration of the external medium, V_0 the initial effective osmotic volume of the cell, A its area (assumed to be constant—a not unreasonable assumption in the case of the biconcave erythrocyte) and k the permeability constant of the erythrocyte for water; that is, a numerical measure of the amount of water that would with unit difference in osmotic pressure between the cell and its surroundings pass through unit surface in unit of time. Since V_0 and A are frequently not accurately known separately, the expression kA/V_0 may for many purposes be used as a secondary constant, k', whose calculated values over a range of concentrations give indication in the same way as do those of k of the applicability of the equations in question.

In the case of hypotonic solutions of non-electrolytes, it has already been shown (Jacobs, 1932) that the observed times of hemolysis over a wide range of concentrations are in fairly good agreement with those predicted by means of the equations, if allowance be made for a decided increase in the "osmotic resistance" of the cells produced by exposure to such solutions. Since there is some reason to believe that this increase in resistance may itself be osmotic in nature, there is no need at present to postulate non-osmotic factors to account fairly well for the observed results with non-electrolytes. In the case of electrolytes, however, which will be discussed in the present paper, conditions are somewhat different. In passing from water through a series of hypotonic solutions of, for example, NaCl of increasing concentration, the properties of the erythrocyte undergo a change, expressed quantitatively by a change in the value of the calculated permeability constant, which seems to depend on other than osmotic factors. Above a certain concentration—roughly 0.02M in the case of NaCl—the behavior of the erythrocyte is in excellent agreement with that predicted by means of the equations; that is to say, a constant calculated value of k' is obtained. Below this point, however, there is a fairly rapid increase in the value of k' with decreasing concentration which ceases only at very great dilutions of the electrolyte. This inconstancy of k', which almost certainly depends upon non-osmotic factors, and which is influenced to a striking extent by the valence of the cations present in the solution, has been very briefly mentioned in a previous preliminary paper (Jacobs, 1930) but has not hitherto been discussed at any length. We believe that it is of possible significance not only in connection with the problem of hemolysis but with certain larger ones having to do with the general question of cell permeability as well.

In the experiments here described, as well as in others omitted for lack of space, the blood used was that of the ox, obtained from freshly slaughtered animals, defibrinated immediately, and kept until needed in a refrigerator. A smaller number of experiments on the blood of man and of several other mammals gave essentially similar results. All observations were made at 20° C. $\pm$ 0.2° with the employment of exactly the same technique as that already described in the fourth paper of the present series (Jacobs, 1932), which may be consulted for further details.

II

In Fig. 1 are presented the results obtained on the same sample of blood with sucrose on the one hand and with NaCl on the other. In order that the results may be strictly comparable osmotically, the observed times of hemolysis are plotted as ordinates, not against the

concentrations of the two solutions, but rather against their freezing point depressions. The latter were calculated by the empirical equation for NaCl:

$$\Delta = 3.6C - 1.3C^2$$

and for sucrose

$$\Delta = 1.86C + 0.2C^2,$$

which for the concentration range actually employed give a fairly satisfactory agreement with published freezing point data.

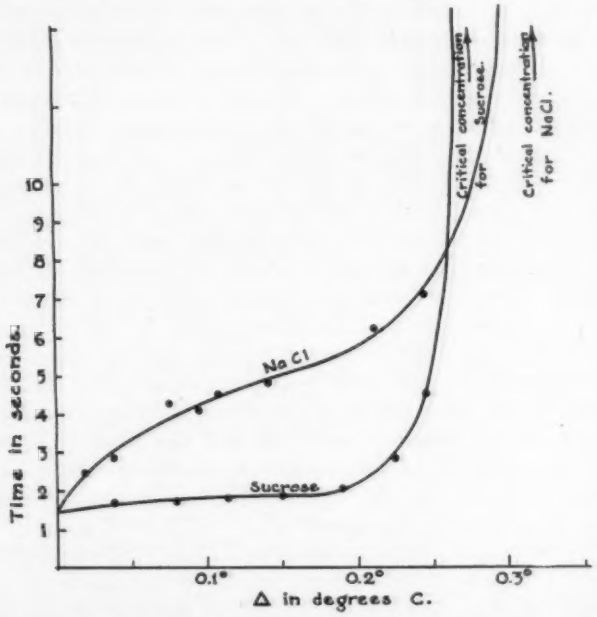

FIG. 1. Rate of hemolysis of ox erythrocytes at 20° C. in solutions of sucrose and of NaCl. One part of blood to approximately 500 parts of solution. Ordinates represent times of 75 per cent hemolysis in seconds and abscissæ calculated freezing point depressions of solutions.

Two things are immediately apparent from the figure. The first is that over most of the range covered by the experiments hemolysis occurs far more slowly in NaCl solutions than in those of sugar of the same osmotic pressure. This difference is especially striking in the most dilute solutions (e.g., of $\Delta = 0.1°$ or less) where the osmotic effect of the solute as calculated by equation 1 is almost negligible, and where the effect actually observed with non-electrolytes is equally insignificant.

but where that found with electrolytes is very pronounced. As will be shown later, this electrolyte effect, which is exerted on the rate of hemolysis rather than on the position of final equilibrium of the system, is especially marked when the valence of the cations present is greater than one.

The second difference between the two curves to which reference has been made briefly above and at greater length in the preceding paper (Jacobs, 1932) is the lower critical hemolytic concentration, *i.e.*, the higher osmotic resistance of the cells, in the case of the non-electrolyte solution. In this particular case the value of Δ for which 75 per cent hemolysis just failed to occur was 0.324° for NaCl and 0.280° for sugar. This effect, which is of the "equilibrium" type, is obviously in the opposite direction from that of the first or "rate" effect, since electrolytes within the range where it is operative tend to favor rather than to oppose hemolysis. Because of the different natures of the two effects, the curves in Fig. 1 cross at a Δ value of about 0.26° for which the time of hemolysis is equal in the two solutions. Above this point there is a relatively narrow concentration range within which hemolysis actually occurs more rapidly in the presence than in the absence of the non-electrolyte. It is to be noted, therefore, that the observed rate of the hemolytic process may be affected by a mere shift in the position of final equilibrium of the system. Similar cases have been discussed elsewhere by the authors (Jacobs, 1928, 1931; Jacobs and Parpart, 1932).

The curve for NaCl in Fig. 1 shows very clearly the general relation between the concentration of a typical electrolyte solution and the time required for it to produce hemolysis; but for a more exact analysis of the extent to which such results are in agreement with osmotic laws it is necessary to employ more strictly mathematical methods. In Table I there have, therefore, been calculated by means of equations 1 and 2 for experiments with NaCl involving three separate samples of blood, values of the constant k', whose meaning is explained above and whose constancy over a given range may be taken as an indication of the applicability for this range of simple osmotic laws. The value of R employed for the calculations in each case was taken as the ratio of the freezing point depression of ox plasma (approximately 0.58° C.) to the freezing point depression of the NaCl solution in which, for the blood in question, the final degree of hemolysis was 75 per cent; this critical hemolytic concentration being determined for each sample of blood by a separate experiment. It was mentioned in the previous paper that a greater constancy of k' is obtained with non-electrolyte solutions if a somewhat smaller value of R than this be employed; but the theoretical justification for this latter procedure is rather questionable, and in the

calculation in that paper of the true permeability constant, k, the same R was used as that here adopted. It should be emphasized that in view of the complexity of the material and of the various simplifying assumptions made in deriving the equations a perfect agreement between theory and observation is never to be expected. For the present, therefore, it seems advisable to use the value of R which is most simply defined and most easily determined, even though a slightly different value may fit the data rather better in some particular cases.

TABLE I

Effect of the concentration of NaCl solutions on the time required for 75 per cent hemolysis of ox blood at 20° C. One part of blood to approximately 500 parts of solution. Each time is the average of four determinations.

Concentration	Δ	Experiment 1 $R = 1.63$		Experiment 2 $R = 1.79$		Experiment 3 $R = 1.69$	
		Time *seconds*	k'	Time *seconds*	k'	Time *seconds*	k'
0.00	0.000	1.35	1.06	1.42	1.34	1.30	1.23
0.005	0.018	2.48	0.62	3.00	0.64	2.28	0.73
0.01	0.036	2.72	0.58	3.75	0.55	2.68	0.64
0.02	0.072	3.60	0.48	4.82	0.48	3.78	0.51
0.03	0.107	4.70	0.41	5.65	0.46	4.68	0.46
0.04	0.142	5.12	0.42	6.15	0.48	5.05	0.48
0.05	0.177	5.65	0.43	6.88	0.49	5.60	0.50
0.06	0.211	6.40	0.44	7.85	0.52	6.68	0.48
0.07	0.246	7.80	0.44	10.62	0.47	7.48	0.53
0.08	0.280	11.02	0.39	35.90	0.20	10.78	0.48
0.09	0.314	30.22	0.20	—	—	130.	0.06

It will be noted in Table I that the value of k' for water alone in all three experiments is relatively high, *i.e.*, 1.06, 1.34, and 1.23, respectively. These values may be compared with those of 1.16 to 1.48 found in the previous paper, when R was similarly determined, for water and for a wide range of concentrations of sugar solutions. It will be further noted that whereas with the non-electrolyte discussed in the earlier paper no appreciable change in k' occurred in passing from water to solutions of a concentration of, say, 0.04M, in the case of NaCl, an enormous change appears on passing to a concentration of only 0.005M; and a further, though much slighter, change by an additional increase in the concentration to 0.01M. Even allowing for the fact that the osmotic pressure of an NaCl solution may be twice as great as that of a sugar solution of the same concentration, it is evident that the striking retardation of hemolysis caused by very dilute solutions of NaCl can scarcely be osmotic in nature.

Passing over the narrow range of concentrations from zero to 0.01M or 0.02M, within which k' undergoes a considerable change in magnitude, we find that for all higher concentrations up to 0.07M or 0.08M the value of k' is not only remarkably constant for a given experiment but that the values obtained with different samples of blood are in good quantitative agreement. It is difficult to believe that the constancy of k' over such a wide range of concentrations is due merely to chance. The most reasonable interpretation of the facts is that within this extensive range the concentration of an NaCl solution is related to the time of hemolysis by simple osmotic laws, as has already been found to be the case (with certain limitations) with non-electrolyte solutions. It is to be noted, however, that the value of the constant for NaCl solutions is only between one-half and one-third as great as for water and for non-electrolyte solutions. The same relation holds for the true permeability constant, k, which, for a given type of blood, is always a definite multiple of k'.

As to the complete lack of agreement between the last value of k' in each series with the remainder, it may be said that determinations of rates of hemolysis in solutions lying so close to the critical hemolytic concentration are notoriously unreliable, as has been pointed out by one of the authors elsewhere (Jacobs, 1928). Successive determinations under such conditions, even when carefully made, show such relatively enormous differences as to render exact quantitative work in this region almost hopeless. It is not unlikely that the very low values of k' at the highest concentrations, where the time of hemolysis exceeds about 10 seconds, may be significant, possibly indicating an escape of salts from the cell with a consequent retardation of hemolysis (see in this connection Ponder and Saslow, 1931); but in view of the difficulty of obtaining accurate data under these conditions we prefer to leave this point unsettled for the present. The important fact remains, nevertheless, that over a wide range the effect of the concentration of NaCl solutions on the rate of hemolysis is in good agreement with that demanded by simple osmotic laws.

III

Turning now to the region of the lowest concentrations (*i.e.*, all below about 0.02M), it is apparent that in this region small changes in the concentration of the electrolyte solution affect the rate of hemolysis in a manner that is not at all in agreement with equations 1 and 2. As a matter of fact, such effects extend to much more dilute solutions than any included in Table I and are, as will now be shown, intimately related to the valence of the cations present.

In Fig. 2 are presented the results of a typical experiment with a single sample of blood in which the time of hemolysis was determined in various hypotonic solutions of NaCl, Na_2SO_4, $CaCl_2$, $MgCl_2$ and $MgSO_4$. Since over most of the range employed osmotic effects must obviously be very slight, actual concentrations rather than freezing point depressions are used in the figure as abscissæ. Furthermore, in order that a wide range of concentrations, including those of a number of extremely dilute solutions, may be covered, the concentrations are

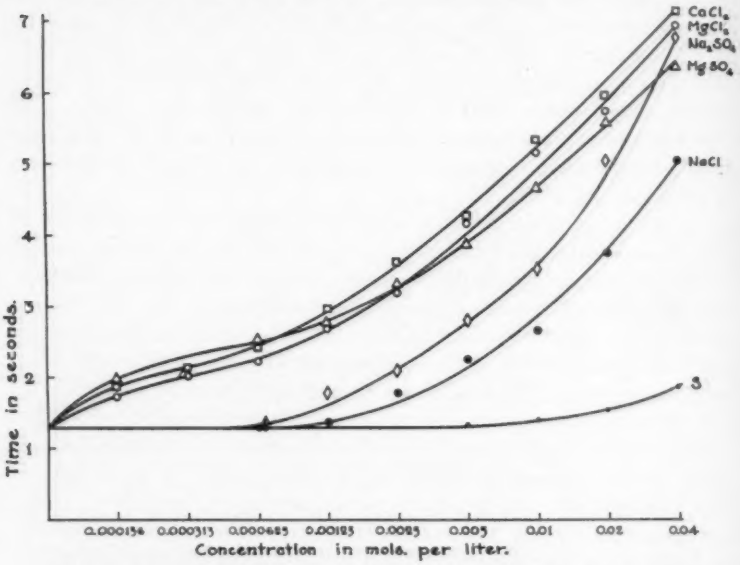

FIG. 2. Rate of hemolysis of ox erythrocytes at 20° C. in solutions of various salts. One part of blood to approximately 500 parts of solution. Ordinates represent times of 75 per cent hemolysis in seconds and abscissæ concentrations of solutions in mols per liter.

plotted logarithmically, *i.e.*, equal distances along the axis of abscissæ are taken to represent equal multiples of concentrations rather than equal arithmetical increments. The figure is therefore comparable with those of Loeb (1922) to which reference will be made below. Included in the figure for comparison is a curve, labeled *S*, which indicates the calculated, and also approximately the observed, effects of sugar solutions having the osmotic pressures of the indicated concentrations of NaCl. It will be noted that the true osmotic effects, which alone are found in such solutions, are entirely negligible over most of the range

covered by the figure and that most of the effects of the electrolyte solutions must therefore be of a different nature.

An inspection of Fig. 2 brings out several additional points of interest. The first is that the salts fall into two sharply-separated groups, both with respect to the concentration at which a visible retardation of hemolysis first appears and with respect to the magnitude of the retardation at any given concentration. Thus, with $CaCl_2$, $MgCl_2$ and $MgSO_4$ a retardation of the order of 0.5 second or 40 per cent is present at a concentration of 0.00015M. A similar retardation is not reached with NaCl and Na_2SO_4 below a concentration of approximately 0.003M, and no detectable effect of any sort is found with either of the latter salts, or with KCl, which was studied in other experiments, below a concentration of about 0.001M. Throughout the entire range employed the relatively greater effectiveness of the salts of Ca and Mg is most marked. With salts of this type the valence of the cation appears to be the factor of chief importance, since there is little difference between $MgCl_2$ and $MgSO_4$.

In the case of NaCl and Na_2SO_4, both of which are rather widely separated in their properties from the salts just mentioned, it would appear that Na_2SO_4 is considerably more effective at a given concentration than is NaCl. This difference is probably to be attributed to the fact that the salt of the dibasic acid furnishes twice as many cations as that of the monobasic acid, the cation being, as already indicated, the ion of chief effectiveness in influencing the rate of hemolysis. If in plotting the two curves the concentrations of the Na ions had been used as abscissæ rather than the molecular concentrations, the curve for Na_2SO_4 would have been shifted to the right by an amount equal to that between two successive indicated concentrations; and in that case the two curves would have almost coincided. In several other experiments, not described here, the times of hemolysis for Na_2SO_4 in the region below 0.01M where osmotic effects are negligible were found to be somewhat below those for NaCl at the same $Na^{\cdot}$ ion concentration. In other words, with the same concentration of $Na^{\cdot}$, SO_4'' at times seemed, if anything, to favor hemolysis as compared with Cl', though it is to be noted that the concentrations of the two anions under these conditions were no longer the same and the differences were at best slight.

In the case of trivalent cations, a number of experiments have been made with $Al^{\cdot\cdot\cdot}$, but the results are too complex to be discussed here, since they involve $H^{\cdot}$ ion effects, agglutination of the erythrocytes, and other complications that have little bearing on the present problem. It may be mentioned, however, that in its ability to retard hemolysis at

very low concentrations, $Al^{...}$, under proper conditions, may very considerably exceed the bivalent ions. With it a distinct retardation of hemolysis is at times obtained at concentrations as low as 0.00001M. The rather complicated nature of the effects of Al salts upon the erythrocyte will be discussed in detail elsewhere.

In addition to the experiments here described, a considerable number of others of the same general type have been performed. Because of the great rapidity of the hemolytic process in water and very dilute solutions, the quantitative accuracy of such experiments is not always as great as might be desired, and there are some slight discrepancies from experiment to experiment; but, on the whole, the results are in very satisfactory agreement and bear out the conclusion here reached, namely, that in dilute solutions cations tend to retard osmotic hemolysis in some non-osmotic manner with an effectiveness that increases greatly with an increase of their valence from one to two, and that anions have comparatively little influence on the process, though in some cases they seem with increasing valence slightly to favor it.

IV

As to the cause of the retardation of hemolysis produced by adding to distilled water electrolytes in concentrations from 0.01M to 0.0001M or even lower, it may be said with a fair degree of certainty that the osmotic pressure of the external solution in such cases is a factor of little or no significance. This is indicated not only by the negligible osmotic effects of such solutions as calculated by means of equation 1, and as actually observed in the case of non-electrolytes, but by the enormous differences in the effectiveness of, for example, NaCl and $MgSO_4$ at the same concentration, or of NaCl and $CaCl_2$ at the same freezing point.

The possibility nevertheless suggests itself that while in such cases the external osmotic pressure is of no importance, there might conceivably be produced by the solutions some indirect osmotic effects on the cells themselves which would influence the rate of the hemolytic process. We have already pointed out (Jacobs and Parpart, 1931) that the erythrocyte is unique among cells in the readiness with which its internal osmotic pressure is affected by apparently insignificant external changes of different sorts. Unfortunately for this explanation, such effects as might conceivably be produced in this way are, in the present case, in the wrong direction. As shown by the difference in the critical hemolytic concentration for electrolytes and for non-electrolytes (see Fig. 1), the "equilibrium" effect of electrolyte solutions is in the direction of favoring rather than of opposing hemolysis. An osmotic ex-

planation of the observed results, either direct or indirect, seems therefore definitely to be ruled out.

A more plausible explanation, because it suggests analogies in both living and in non-living systems, is that the rate of entrance of water into the erythrocyte is affected by low concentrations of ions in a manner similar to that observed by Lucké and McCutcheon (1929) in the case of the *Arbacia* egg and by Loeb (1922) in the case of collodion-gelatin membranes on the alkaline side of the isoelectric point of the gelatin. The former workers have reported that cations inhibit the passage of water into the *Arbacia* egg to an extent which increases with their valence, while anions behave in the opposite manner. In the case of collodion-gelatin membranes, where the factors concerned are obviously of a very simple physico-chemical nature, the results obtained are much the same; the nature of these effects has been discussed at length by Loeb. The erythrocyte differs from both the *Arbacia* egg and the artificial membrane in the much less prominent, and indeed somewhat doubtful, effect upon it of anions as compared with cations; but the striking difference between the ions of the alkali metals, on the one hand, and those of the alkaline earths on the other is found in all three cases, and may conceivably be due to the same causes.

An alternative explanation is that the effect of ions is on the rate of escape of hemoglobin from the cell rather than on the rate of entrance of water into it (see in this connection the discussion by Jacobs and Parpart, 1932, of the effect of narcotics on hemolysis). This explanation, however, while not completely ruled out by the existing evidence, seems to us to be less probable than the other one in view of the fact that the "equilibrium" effect of electrolytes on hemolysis, unlike that of narcotics, is in the opposite direction from the "rate" effect. Whatever the explanation of the effect of traces of electrolytes on the rate of hemolysis may ultimately prove to be, however, the observed facts are themselves entirely definite; and the non-osmotic factors shown to be concerned in the process would seem to be worthy of consideration in connection with theoretical discussions of the nature of cell permeability.

Summary

1. In NaCl solutions of concentrations from about 0.02M to 0.07M or 0.08M the rate of hemolysis of ox blood is related to the concentration of the solution as if the process were governed by simple osmotic laws.

2. The permeability constant for water over this range is between one-half and one-third as great as that previously found for non-electro-

lyte solutions. At concentrations below 0.02M the calculated "constant" changes with the concentration of the solution in a manner indicative of the presence of non-osmotic factors of some sort.

3. The retarding effect upon hemolysis of dilute solutions of electrolytes increases rapidly with the valence of the cations present. The valence of the anions is much less important but, if anything, acts in the opposite sense.

4. The tentative suggestion is offered that under certain conditions ionic forces may modify to an appreciable extent the rate of the osmotic intake of water by the erythrocyte.

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HIBERNATION AND DIAPAUSE

Physiological Changes During Hibernation and Diapause in the Mud-dauber Wasp, Sceliphron cæmentarium (Hymenoptera)

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It has been known for some time that many organisms enter periods of inactivity during winter or upon exposures to low temperatures. In some cases it has been clearly demonstrated that periods of rest or diapause are quite independent of external temperatures for their occurrence. The parts played by heredity and environment in these phenomena have also been much discussed. Quantitative physiological observations on single individuals during these periods of quiescence, however, have been carried out on but few forms and particularly is this true for lower forms, especially the insects (Uvarov, 1931; Dreyer, 1932; Ashbel, 1932, etc.). The present paper is concerned with results of a detailed study of certain physiological changes taking place during the developmental life cycle of the common yellow-legged mud-dauber wasp, *Sceliphron cæmentarium*.

The mud-dauber wasp, *S. cæmentarium*, is extremely favorable material for physiological investigations since it can be readily obtained in its developmental stages in large numbers and is easily kept in the laboratory with a minimum of care. Its life-cycle is relatively simple. After completion of the mud nest, spiders are captured, paralyzed, and put into the individual cells of the nest. A single egg is laid by the female wasp on the abdomen of the first spider introduced. Other paralyzed spiders are added to the cell as food for the developing larva and the cell is then sealed. The egg hatches in a very few days (depending on external temperature) and the larva eats voraciously of the enclosed spiders and quickly attains the stage at which it spins a cocoon about itself. Within this cocoon case the animal goes through the remainder of its larval, prepupal and pupal life and eventually emerges as an adult wasp. The length of the larval stage is of considerable interest, since, normally, animals hatching late in the season (August–September) hibernate in this stage. Larvæ from eggs laid early in the summer (June–July), in most instances, do not go through hibernation in the larval stage but develop uniformly, emerging out-of-doors in

from 19 to 25 days (Rau, 1918). It is reasonable to assume that two varieties are normally produced—one that goes through development from egg to adult at a fairly uniform rate with no marked periods of cessation—another that normally hibernates out-of-doors in the larval stage and thus does not develop during such periods. Since it is almost impossible to collect all the eggs laid by a single female wasp during the entire laying period, one cannot say definitely that both types of eggs are laid by the same individual. Indirect evidence would seem to indicate, however, that eggs laid early in the season invariably go through development without a cessation while those laid later in the season are usually of the diapause type. A somewhat similar observation for the codling-moth has been reported by Glenn (1922), Shelford (1927), and others. Both types of individuals have been obtained in Iowa as well as from New Jersey, Pennsylvania, Maryland, and Texas. Experimental materials for this investigation were taken over a period of three successive years.

The procedure followed in obtaining animals was as follows: The mud-nests were collected at intervals throughout the year and larvæ in various stages of their developmental life cycles, as far as diapause was concerned, were thus obtained. The animals were completely removed from the cocoon cases and kept separately in shell vials or gelatin capsules at known constant temperatures throughout the experiments. Eggs laid early in the season (July) were obtained at the time of laying and after hatching were fed paralyzed spiders taken from the nests. The larvæ in these experiments were kept at constant temperatures and grew in quite a normal fashion. It was thus possible to obtain in this manner accurately timed organisms for comparison with those taken at random. Inasmuch as the last larval stage is a non-feeding one the organism is relatively easy to keep under laboratory conditions. Body weights and morphological and physiological histories were kept for individual larvæ. Oxygen consumption was determined by the modified Krogh manometer (Bodine, 1929). But one larva was used at a time and this always in the same manometer throughout the period of the experiment. Some 225 to 250 larvæ have been individually studied. Inasmuch as the results obtained are qualitatively similar it seems desirable to express them graphically. This method shows most clearly the general course of the physiological and other changes followed by the larvæ throughout their development.

Experimental Results

I. *Non-diapause Type of Organism*

Oxygen consumption and body weight changes during the entire

developmental life cycle of non-diapause individuals have been studied. All results obtained have been qualitatively similar so that only typical cases will be presented. At 28° C. the length of time required for the entire development from laying of egg to emergence of wasp is approximately 25 to 28 days. The oxygen consumption of the egg during development steadily increases up to the time of hatching. During the active feeding and growing periods of the larva, body movements are so marked that satisfactory measurement of the rates of oxygen consump-

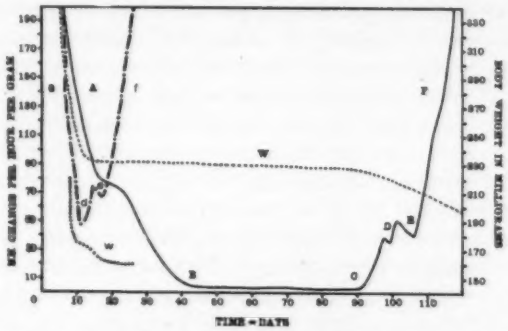

FIG. 1. Rates of oxygen consumption and body weights during the development of diapause and non-diapause types of animals at constant temperature of 28° C. Ordinates at left, millimeters change of manometer fluid per hour per gram organism (same manometer used throughout experiment; to convert readings into actual amounts of oxygen, results are multiplied by factor for the manometer in question). Ordinates at right, body weight in milligrams. Abscissæ, time in days indicated. Letters indicate different periods in life cycle of organisms. Large letters for diapause type, small ones for non-diapause type. A = spinning of cocoon. B = beginning of diapause. C = end of diapause. D = formation of prepupa. E = pupation. F = pigmented pupa ready to emerge. W = body weight. —·— = oxygen consumption, non-diapause type. ———— = oxygen consumption, diapause type. · · · · · = body weight, non-diapause type. - - - - - = body weight, diapause type. For further description, see text.

tion can not be obtained. After the spinning of the cocoon, the animal becomes less active and it is largely for this reason that the results presented in this paper begin at this point in the organism's development. Prior to and during the spinning of the cocoon, the alimentary canal is emptied of waste materials and this too adds much to the desirability of beginning measurements of oxygen consumption rates and changes in body weights at this stage.

In Fig. 1 the rates of oxygen consumption and body weights of a typical non-diapause type individual are graphically shown. From an inspection of this figure it will be noted that the rates of oxygen consumption during the spinning of the cocoon are at first high but after

spinning they quickly drop to a minimum. This minimum value is at the time the animal reaches the prepupal stage. After the prepupal stage an increase in the rate of oxygen consumption occurs, during which the animal prepares for pupation. During pupation a drop in rate of oxygen consumption again takes place and this is followed by a steady and marked increase up to emergence of the wasp. This drop in oxygen consumption rate during pupation, or the so-called U-shaped oxygen consumption curve, is quite characteristic for this phenomenon since it has been reported for many other forms undergoing complete metamorphosis (Taylor and Steinbach, 1931, and others). The prepupal drop in the oxygen consumption curve is equally characteristic but seems to have been reported for but few forms (Fink, 1925). It is thus seen that during the development of the wasp definite cycles or rhythms in rates of oxygen consumption occur which are closely correlated with the morphological stages through which the animal passes. Changes in body weight are also of considerable interest (Fig. 1, curve *w*). During the spinning of the cocoon a rather marked drop in weight occurs, due largely to the emptying of the alimentary canal. After this, loss in body weight is gradual but continuous up to the time of emergence.

II. *Diapause Type of Organism*

As noted above, eggs laid late in the summer (August–September) usually produce animals showing a diapause. These organisms are quite similar to the non-diapause variety in their development, the most striking difference being the length of time necessary for development at constant high temperatures (20–35° C.). In Fig. 1 there is graphically represented for a diapause individual rates of oxygen consumption and body weights during developmental stages comparable to those described above for the non-diapause organism. From an inspection of these curves it will be noted that a marked decrease in rate of oxygen consumption down to a minimum value occurs during the spinning of the cocoon and in preparation for diapause. This minimum value at constant high temperatures (20–35° C.) is always considerably lower than that reached by the non-diapause form even though the morphological stages are similar in both cases. During diapause at constant high temperatures (20–35° C.) minimum rates of oxygen consumption are practically constant and at 28° C., as shown in Fig. 1, last some 40 to 50 days. As noted further on, this minimum rate of oxygen consumption and its duration are modified to a considerable degree by different temperatures. Changes in body weights in diapause organisms are qualitatively similar to those undergone by non-diapause individuals, the

only difference being a marked period of almost constant body weight during diapause.

In general, the physiological changes through which both types of organisms pass during development are, with the exception of diapause, quite similar. The rhythmic or cyclic changes correlated with larval, prepupal, and pupal changes are strikingly indicated in both. The diapause individuals, at 28° C. (as indicated in Fig. 1), require from five to six times longer for their development. Questions as to the total amounts of energy involved in the development of the two types of individuals are of considerable interest, but since it is almost impossible to secure all the eggs from the same female, many uncontrollable factors enter which tend to make such calculations hazardous. Differences in initial body weights, amounts of food stored in nests, and similar conditions make absolute comparisons, as far as the total oxygen consumed and number of days required for development, impractical for this form. Similar experiments carried out on the eggs of the silkworm by Ashbel (1932), however, show that the amounts of oxygen consumed and the number of days required for development are more or less constant for diapause and non-diapause eggs.

III. *Reactions of Diapause to Temperature*

Inasmuch as diapause seems independent of external temperatures for its occurrence it was thought desirable to determine what the effects of different temperatures would be on its duration and intensity. Rather extensive experiments have been carried out using diapause type animals taken from out-of-door environments and determining their rates of oxygen consumption throughout hibernation and growth periods. In addition, organisms in similar physiological states have been experimentally subjected to controlled temperatures and their responses studied.

1. *Diapause animals under out-of-door temperatures.*—Diapause larvæ in the same morphological stages were collected from nests during different periods of the year, from August to April, put at constant temperature (28° C.) and their rates of oxygen consumption and growth followed. Results, typical of such experiments, are graphically indicated in Fig. 2. An inspection of this figure shows that animals collected late in August have rather marked periods of low oxygen consumption rates (diapause) similar to those pointed out previously for diapause animals kept continuously at constant high temperature (Fig. 1). With the approach of low out-of-door temperatures in November the length of low oxygen consumption rates (diapause) at 28° C. gets progressively shorter. Animals put at 28° C. late in December, after

being exposed to rather long periods of low out-of-door temperatures, show no periods of low oxygen consumption rates but develop in quite a uniform and normal fashion. That this gradual shortening of the low oxygen consumption rate (diapause) is not due to the animals being of different developmental ages when collected at different periods of the year, can be easily demonstrated. Individuals taken under identical environmental conditions early in the fall and kept out-of-doors under observation for the remainder of the year always give progressively shorter and shorter periods of low oxygen consumption rates the later they are put at constant high temperature (28° C.). In other words, exposures to low out-of-door temperatures during winter in some way

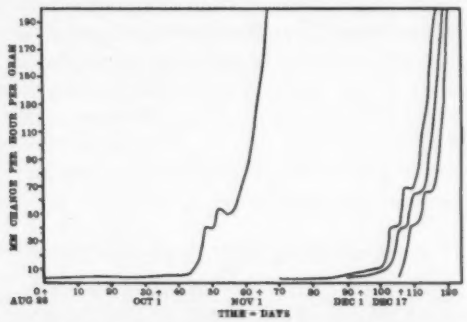

FIG. 2. Rates of oxygen consumption for diapause-type animals collected out-of-doors at different periods during year and then placed at 28° C. until emergence. All animals in same morphological stage (hibernating larval stage) at time of collection. Ordinates, millimeters change of manometer fluid per hour per gram of organism (see note under Fig. 1). Abscissæ, time of year and days indicated. Each curve represents oxygen consumption for a single larva. For further description, see text.

or other shorten the length of diapause or diapause progresses during exposures to low temperatures. No morphological or developmental changes are evident in the animals during the periods of low oxygen consumption rates (diapause).

2. *Diapause animals under controlled temperatures.*—Animals of known history, as far as diapause was concerned, were collected in large numbers and put at constant temperatures of 2° C. where they were left for varying periods. At different intervals of time some were taken from 2° C. and put at 28° C. and left there until emergence of the adult animal. Rates of oxygen consumption and development were carefully studied on animals thus treated. Figure 3 shows, graphically, results typical of such an experiment. From an examination of this figure it

will be noted that exposures of diapause animals to 2° C. cause a marked shortening of the period of low oxygen consumption rates (diapause) when the organism is subsequently transferred to 28° C. The degree to which this period is shortened is conditioned more or less quantitatively by the length of exposure to 2° C. Short exposures (5–10 days) cause but little change in duration while long exposures (50 + days) cause complete disappearance of the period. No morphological changes can be noted in the organisms kept for some time at 2° C. If the exposure is too long (over 3 to 4 months) a rather high mortality results. This, however, is a much longer time than necessary for com-

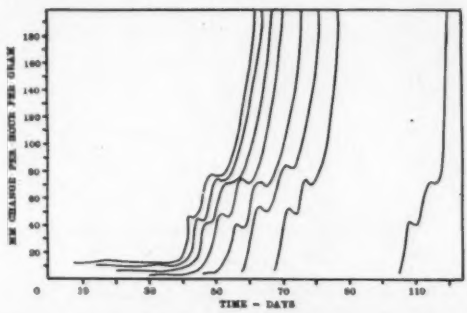

FIG. 3. Rates of oxygen consumption for diapause-type animals kept at 2° C. for varying periods and then put at 28° C. until emergence. All animals collected on same date at beginning of experiment and in same morphological stage (hibernating larval stage). Ordinates, millimeters change of manometer fluid per hour per gram of organism (see note under Fig. 1). Abscissæ, time in days indicated. All oxygen curves begin on day animal was transferred from 2° to 28° C. Period of exposure to 2° C. indicated by number of days from start of experiment to time oxygen curve begins. Each curve represents oxygen consumption for a single larva. For further description, see text.

plete disappearance of the period of low oxygen consumption rates (diapause). It is thus evident from such results that diapause progresses or is influenced by exposure to low temperatures (2° C.) and that such action of low temperatures out-of-doors must be a factor in the normal reaction of the organism to its environment. Temperature seems to have little influence on the occurrence of diapause but it is unquestionably a factor for its duration.

In another series of experiments, larvæ just entering diapause were collected from out-of-doors and put at constant high temperatures (35, 28, 25° C.) as well as at 2° C. For those kept at the higher temperatures, oxygen consumption rates were determined during the entire developmental stages. In the case of those at 2° C., individuals were

taken at different time intervals and transferred to 25° C., at which temperature their oxygen consumption rates and development were followed. Particular attention was given to the time necessary for diapause to disappear (as judged by the lack of low oxygen consumption rates at 25° C.) at this low temperature (2° C.) so that a relative comparison between the length of diapause at the different temperatures could be made. Figure 4 indicates graphically typical results of such a series of experiments. An examination of this figure shows that low

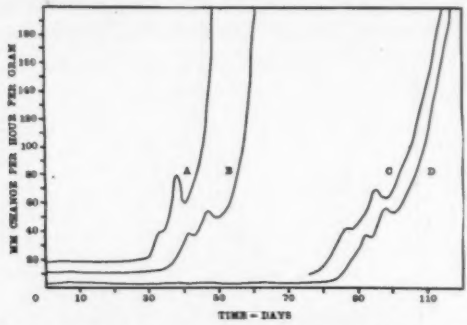

FIG. 4. Rates of oxygen consumption for diapause-type animals kept at constant temperatures as well as for those kept at 2° C. and subsequently transferred to 25° C. All animals collected at same time and in same morphological stage (hibernating larval stage). Ordinates, millimeters change of manometer fluid per hour per gram of organism (see note under Fig. 1). Abscissæ, time in days indicated. Each curve represents oxygen consumption for a single larva. Curve *A*, animals kept at constant temperature of 35° C. from beginning of experiment until emergence. Curve *B*, animals kept at 28° C. from beginning of experiment until emergence. Curve *C*, animals kept at 2° C. from beginning of experiment and then transferred and kept at 25° C. until emergence. Curve shown indicates period of exposure to 25° C. Period of exposure to 2° C. indicated by number of days from start of experiment to time oxygen curve begins. Curve *D*, animals kept at 25° C. from beginning of experiment until emergence. For further description, see text.

rates of oxygen consumption (diapause) occur at the different temperatures (25–28–35° C.). Of particular note is the relative length of diapause at these temperatures. At 35° it took approximately 33 days, at 28°, 35 days and at 25°, 85 days. A temperature coefficient of approximately 2.5 has been found for the duration of diapause at these constant high temperatures. In the experiments at 2° C. it took approximately 75 to 80 days for diapause, which is about equal to the time required at 25° C. In all experiments it has been found that low temperatures (10–2° C.) are almost as efficient for the progress of diapause as are temperatures as high as 25° C. It is thus evident from these results that diapause occurs independently of the external temperature but that

its duration is to a marked degree conditioned by temperature. The most unusual nature of the effect of temperature on it is the fact that low temperatures, much below the threshold values for development for this species, affect it in a marked degree.

The relative rates of oxygen consumption at different temperatures are also of considerable interest. At 28 and 35° C. the average rates

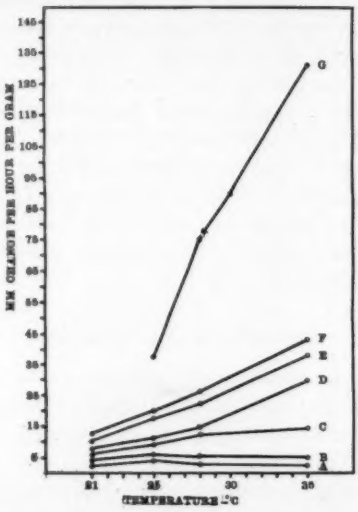

FIG. 5. Effect of temperature on rates of oxygen consumption of diapause-type animals at different periods of life cycle. Ordinates, millimeters change of manometer fluid per hour per gram of organism (see note under Fig. 1). Abscissæ, temperature, degrees centigrade. Curve *A*, animal at beginning of diapause. Curve *B*, animal in middle of diapause. Curve *C*, animal at end of diapause. Curve *D*, animal coming out of diapause entering prepupal stage. Curve *E*, animal in prepupal stage entering pupal stage. Curve *F*, animal in pupal stage. Curve *G*, animal ready to emerge. For further description, see text.

expressed in terms of millimeter changes in manometer fluid per hour per gram organism are 18 and 12 respectively, while at 25° C. they are 3. At 2° C. rates are so low that it is practically impossible to get satisfactory results over long periods of time. At temperatures of 25° C. and below, rates of oxygen consumption are always extremely low in comparison with those at temperatures above this value. Such marked differences are, perhaps, due to a regulatory mechanism which enables the animal under out-of-door conditions to better meet and endure extreme fluctuations of temperature.

During diapause, however, responses to short exposures to different constant temperatures are quite in contrast to those for organisms in which diapause has ceased. In Fig. 5 there is graphically represented the effects of different temperatures on the oxygen consumption rates of organisms in different stages of diapause. From an inspection of this figure it can be readily seen that marked responses are given only after the period of diapause is well advanced. When reactions to different temperatures are first noted they are of small magnitudes (Fig. 5, *C–D*). As diapause progresses and begins to wane progressively greater responses are given. Curiously, temperature coefficients for the different periods are quite similar even though the actual amounts of oxygen are of greatly different magnitudes. During diapause the organism is apparently quite dependent on the particular physiological state at which it happens to be for its response to different temperatures. In early periods it is less responsive than later ones and only approaches the response normally given by non-diapause animals after the effects of diapause have disappeared.

Summary and Conclusion

1. Rates of oxygen consumption and changes in body weight during the development of diapause and non-diapause types of the mud-dauber wasp, *Sceliphron cæmentarium*, at constant temperatures are presented and compared.

2. Diapause seems, for its occurrence, independent of environmental temperatures, but its duration is conditioned to an appreciable degree by these factors.

3. Cyclic or rhythmic changes in oxygen consumption and body weight during the developmental life cycle of the wasp are pointed out.

4. Quantitative responses to temperature are modified to an extreme degree by the diapause phenomena.

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THE RESPIRATORY GAS EXCHANGE IN TERMOPSIS NEVADENSIS

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The series of studies made in recent years by the members of the Department of Zoölogy at the University of California, as well as other investigations, have thrown much light on the relations between environmental factors and the habits of termites, particularly with respect to diet, moisture, and symbiotic microörganisms. Questions have arisen at times in connection with other ecological problems pertaining to the utilization of and the dependence on oxygen by the termite colony. In particular, it is conceivable that small differences in the oxygen tension of various habitats might influence the distribution of species and life cycle of individuals. In considering the matter from the ecological point of view, one is confronted by the difficulty that very little is known about the normal respiration of the termite itself. It was to obtain information concerning this point that the present study was made.

Several hundred specimens of *Termopsis nevadensis,* secured from the vicinity of Santa Cruz, California, were kept in open dishes, and fed a combination of moist filter paper and wood scraps. For experimental work they were sorted roughly into sizes, use being made of only the nymphs. Although such is probably not the case, there is a possibility that the respiration of the soldiers and reproductives might differ from that of the nymphs. Both oxygen consumption and carbon dioxide production were measured manometrically by the Warburg method. Usually the gas exchange of about twelve termites at a time was measured in each manometer vessel. The results were sufficiently consistent to indicate that variations between individuals were thereby eliminated. In most cases the termites were weighed and the results expressed as cubic millimeters of oxygen absorbed or carbon dioxide evolved per gram per hour. Occasionally, however, this was not necessary, particularly when the results were purely comparative in nature.

It is, of course, very difficult to keep the termites motionless and otherwise achieve basal conditions. This must be constantly borne in mind in determining respiratory quotients. Cleveland (1925) has reported a quotient of 1.0, but since he does not state the conditions under which the measurements were made, it is to be assumed that he was deal-

ing with normal, active animals, feeding on a preponderantly carbohydrate diet, animals which would be expected to show such a quotient. However, in investigating the respiration of invertebrates, both anaërobic and aërobic, it is not necessary to achieve that particular basal state demanded in a mammal, provided the conditions are uniform throughout the entire series of experiments. With termites these conditions may be attained satisfactorily, as is shown by the following experiment.

Experiment 1.—The respiration of ten groups of termites with varying number of individuals in each was measured at 32° C. The oxygen consumption in cubic millimeters per gram per hour was: 930, 820, 850, 740, 740, 830, 760, 750, 770, 860. The extreme variation is about 20 per cent.

The possibility should not be overlooked, in dealing with large numbers of termites of somewhat different ages, that the respiratory rate may vary with age. The possibility is eliminated, however, as indicated thus:

Experiment 2.—The oxygen consumption of two groups of termites was measured at 20° C., the first group consisting entirely of individuals of an average length of 10 millimeters, the second of 15 millimeters. The consumption of Group I was 433 cu. mm. O_2/gram $\times$ hour and that of Group II was 413 cu. mm. O_2/gram $\times$ hour, the difference lying within the experimental error.

The general problem of the relation between oxygen tension and oxygen consumption has been the subject of numerous investigations. Without entering at this point into any detailed discussion of the results of this work, it may be stated that there has not yet been found any clear correlation between the two. Some animals are able to acquire and use their normal amount of oxygen at extremely low tensions; others are very closely dependent upon the tension. The characteristic reaction of each species must be determined experimentally in every case. (For reviews of the literature, reference may be made to papers by Helff, 1928, and Hyman, 1929.)

Experiment 3.—To ascertain the general relation between oxygen tension and consumption a series of comparative measurements were made with several groups of animals at 20° C., using oxygen concentrations of 100, 21, 10, 5, 2, and 0.8 per cent. For the lowest tension a tank of commercial nitrogen served with oxygen present to the extent of 0.8 per cent as an impurity. The procedure in all cases was to establish the normal rate in air and then to replace the air in the manometer vessel with the gas mixture to be investigated. Frequently two or more mixtures were introduced successively and when this was done the order was varied from group to group. Check readings were made at

the end in air to insure that no injury has been done the respiratory mechanism through subjection to low or high oxygen tensions. No abnormalities were observed save in the case of 0.8 per cent. The rate in air after exposure to this tension was a little low but soon rose to the normal. The initial low rate was probably due to the immobility of the animals which is induced by very low tensions. To the immobility

TABLE I

Relative Oxygen Absorption of Termopsis at Varying Oxygen Tensions

Group no.	Oxygen concentration	Oxygen absorption
	per cent	*per cent of normal*
I	100	111
II	100	93
III	100	86
IV	100	82
I	21	100
II	21	100
III	21	100
IV	21	100
V	21	100
VI	21	100
I	10	88
II	10	93
I	5	86
II	5	83
III	2	64
IV	2	73
V	0.8	26
VI	0.8	30

may also be partially ascribed the low respiratory rate *during* exposure to 0.8 per cent oxygen. Since all these rates are relative and are compared to the rate in air as normal, they may be expressed on a percentage basis, taking the rate in air as 100 per cent. The data are summarized in Table I. It will be noted that as the tension decreases, the relative oxygen consumption also decreases, but not proportionally.[1] The respi-

[1] The effect of pure oxygen appears to be slightly inhibitory. This phenomenon has been observed in the case of other organisms but has never been satisfactorily explained. A suggestion which might be advanced is that the oxygen at high tension inhibits the respiration of the microörganisms of the gut. This fauna is killed by prolonged exposure to oxygen and may be sensitive to shorter exposures.

ration remains very near the normal until tensions below 5 per cent are reached and even with less than 1 per cent oxygen the respiration is almost one-third of its usual value. This situation indicates a very high degree of independence of the oxygen tension on the part of the termite. Furthermore, there appears to be a very well-developed capacity for utilizing extremely small quantities of oxygen. This capacity was made evident in the above experiment when the gas used was 0.8 per cent oxygen in nitrogen. For here the rate of respiration underwent a steady decrease, indicating that the oxygen tension was being materially reduced by the termites themselves and that the rate decreased along with the tension.

It seemed advisable to investigate more thoroughly this ability of the termites to utilize very small concentrations of oxygen. Therefore a series of studies was made, the data for which are consolidated in Table II. For the purpose of orientation a group of normal termites was first used with a mixture containing 0.7 per cent oxygen and the gas exchange followed for several hours (data not shown in Table II). The oxygen uptake was steady for a short time (about 30–40 minutes), then began to fall off. The decrease continued until the gas consumption ceased. But on continuing the readings it was observed that a positive pressure appeared, suggesting that now some other gas was being evolved. The rate of evolution became constant within an hour and remained so as long as measurements were continued—a matter of several hours. Since the vessel contained strong potassium hydroxide, this gas could not be carbon dioxide. The experiment was repeated with an inset of 10 per cent sulphuric acid as well as alkali, but the general course of the reaction was similar. The termites therefore evolve a gas which can be absorbed by neither acid nor alkali. No further attempt was made to determine the exact composition of this substance, but there is a strong possibility that it may be hydrogen or methane, or a mixture of both. If so, a reasonable assumption is that the microorganisms in the gut are responsible. The principal constituent of the termite diet is cellulose and the breakdown of this material is usually ascribed to the protozoa and possibly bacteria which inhabit the digestive tract. In other animals which utilize cellulose in a similar manner, such as cattle, large amounts of hydrogen and methane are produced. There is therefore considerable likelihood that we are dealing with an analogous situation in the termite, although naturally such a statement cannot be made with certainty in the absence of a quantitative analysis of the gas produced.

In order to investigate the rôle of the intestinal fauna in the produc-

tion of this material—which we may call for lack of a more exact description the "undetermined" gas—a number of termites were de-

TABLE II

Oxygen Absorption by Termites at Very Low Tensions

A. Group	I	II	III	IV	V	VI	VII
B. Previous treatment of termites	Normal	Normal	Normal	Defaunated	Defaunated	Defaunated	Defaunated
C. Initial O_2, per cent	0.7	0.7	0.7	0.7	0.7	2.08	2.08
D. Weight in mg	513	1067	457	552	1126	386	699
E. Duration of experiment in hours	6	6⅝	7¾	6½	6	10½	6½
F. Net gas exchange in cu. mm.	−0.5	+52	−4.5	−83	−88	−333	−271
G. Rate of production of undetermined gas in cu. mm. per hour	11.3	19	9	0	0	0	0
H. Total production of undetermined gas in cu. mm.	79	130	77.5	0	0	0	0
I. Rate of production of undetermined gas in cu. mm. per gram termite per hour	22.1	17.8	19.7	0	0	0	0
J. Total gas in manometer vessel in cu. mm.	13,150	12,650	13,200	13,110	12,450	15,250	12,850
K. Oxygen at beginning of experiment in vessel in cu. mm. J × C	92	88	92	92	87	318	270
L. Total oxygen absorbed in cu. mm. F − H	79.5	78	74.5	83	88	333	271

faunated. This was done by the method of Andrew (1930) which consists of the application of oxygen at several atmospheres pressure. When defaunated termites are placed in 0.7 per cent oxygen there is no indication whatever that any gas is evolved. The oxygen consumption

sinks to zero and remains there indefinitely. It seems legitimate therefore to ascribe the inert gas production to the intestinal fauna.

The complicating factor of formation of the undetermined gas may thus be eliminated by defaunation, but the question is introduced whether defaunated and normal termites react in the same way to low oxygen tensions. To investigate this matter and simultaneously to secure data concerning the original problem of utilization of small amounts of oxygen the following experiment was performed.

Experiment 4.—Seven groups of termites were placed in low concentrations of oxygen. Groups I, II, and III were normal, the remainder were defaunated. The first three groups and also Groups IV and V were placed in 0.7 per cent oxygen and Groups VI and VII in 2.08 per cent oxygen. The oxygen content of these mixtures was checked carefully by analysis with the Haldane gas apparatus. Then the gas exchange in each group was measured until, in the case of the first three groups, the rate of production of the undetermined gas was clearly established, and in the case of the others the oxygen consumption had ceased for at least two hours. At the end of this time the readings were discontinued and the *net gas exchange* of each group calculated. This, of course, is obtained from the initial and final manometer readings (see line F in Table II). With the defaunated groups the net exchange is equal to the total oxygen absorption, since there is no other gas concerned. With the normals the net exchange is equal to the oxygen absorbed plus the undetermined gas evolved. To find the total oxygen absorption we must subtract from the net exchange the total undetermined gas evolution. This involves the fairly reasonable assumption that the production of undetermined gas has the same constant value while oxygen is being taken up that it is observed to have after oxygen uptake ceases. This assumption, though reasonable, still remains an assumption, for definite evidence cannot be secured until a method is devised for differentially absorbing the undetermined gas in the vessel as fast as it is formed. There appears to be no method at present for doing this. If we, then, subtract the total undetermined gas evolved from the net gas exchange, we get the probable total oxygen consumption of the normal groups (see line L in Table II). Finally, since the volume of the vessels is known and also the percentage composition of the gas initially introduced, the actual initial quantity of oxygen can be calculated (see line K in Table II). The total oxygen usage may then be compared with the total oxygen available.

From the data presented in Table II the following conclusions may be drawn. In the defaunated groups (IV–VII inclusive) there is a fairly close correspondence between the oxygen absorbed and the

amount initially present in the closed system. At least it may be stated that there is no significant quantity of oxygen remaining in the system when the oxygen consumption of the termites ceases. This appears to be true irrespective of the initial concentration (compare Groups IV and V with VI and VII). In the normal termites there seems to be a slight residue of oxygen, a concentration of the order of 0.1 per cent or a tension of less than one millimeter of mercury. But this difference between the normal and defaunated animals is too slight to be of significance, particularly since (1) the difference is of the order of the experimental error (as indicated by the deviations in Groups IV–VII) and (2) the assumption of a constant rate of inert gas evolution, irrespective of oxygen tension, may not be precisely consistent with the facts. In general, however, it is possible to state that both normal and defaunated termites are able to utilize substantially all the oxygen in the immediate environment even though the latter reaches exceedingly low tensions.

Under anaërobic conditions at least the production of undetermined gas is very constant (see line I in Table II) at a rate of about twenty cubic millimeters per gram termite per hour. A further study of this gas production would be of interest with respect to the composition of the gas and also its possible bearing on the problem of cellulose digestion.

The results obtained with low oxygen tensions suggest further questions: (1) What is the aërobic carbon dioxide production in normal and defaunated animals? This involves also the determination of the respiratory quotient of both types of animal. (2) Is carbon dioxide produced anaërobically, and if so, can the termites incur an oxygen debt? (Such has been found to be the case with the cockroach by Davis and Slater, 1926, 1928.)

Experiment 6.—Eight groups of termites were investigated (see Table III). Groups I–IV were normal animals. Groups V–VII were defaunated one day previously and Group VIII was defaunated two weeks previously. In every case the gas exchange was determined in air in the presence of 5 per cent KOH (line C, Table III) and then in air without alkali (line D, Table III). When no gases other than oxygen and carbon dioxide are involved the result of the first determination represents the oxygen consumption, since the carbon dioxide is quantitatively absorbed by the KOH. The observed gas exchange in the second determination then represents the difference between the oxygen consumption and carbon dioxide production. Since the former is known, the latter may be calculated (line E, Table III). It was ascertained in the previous experiment (Experiment 5) that there is no pro-

duction of the undetermined gas by defaunated termites and therefore in Groups V–VIII the observed exchange of carbon dioxide and oxygen may be taken as the corrected, or true, exchange (lines H and I, Table III). With normal termites it may be assumed, as previously, that

TABLE III

Carbon dioxide production and respiratory quotient of termites. Gas exchange in all cases expressed as cubic millimeters per gram termites per hour.

A. Group and condition	I Normal	II Normal	III Normal	IV Normal	V Defaunated 1 day	VI Defaunated 1 day	VII Defaunated 1 day	VIII Defaunated 2 wks.
B. Weight in mg.	731	633	681	866	852	648	977	694
C. Observed oxygen consumption in air (KOH)	186	218	173	239	190	239	154	200
D. Observed gas exchange in air (no KOH)	11	14	21.5	22.5	−3.5	2.0	4.0	−36
E. Observed carbon dioxide production (C–D)	192	232	194.5	261.5	186.5	241	158	164
F. Observed R.Q.	1.06	1.065	1.12	1.095	0.98	1.005	1.025	0.82
G. Production of undetermined gas (average from Table II)	20	20	20	20	0	0	0	0
H. Corrected oxygen consumption in air (KOH) C–G	206	238	193	259	190	239	154	200
I. Corrected carbon dioxide production in air (no KOH) C–D	197	232	194.5	261.5	186.5	241	158	164
J. Corrected R.Q.	0.955	0.975	1.005	1.01	0.98	1.005	1.025	0.82
K. Total gas production in nitrogen	46.5	52	48.5	48	50.5	55.5	43	43.5
L. Carbon dioxide production in nitrogen (K–G)	26.5	32	28.5	28	50.5	55.5	43	43.5

under aërobic conditions there is a constant production of about twenty cubic millimeters of the undetermined gas per gram termite per hour; otherwise the respiratory quotient of the normal animals appears to be considerably over unity (line F, Table III). This factor may be al-

lowed for and corrected values of the oxygen and carbon dioxide exchange obtained (lines H and I in Table III). Applying this correction, the values of the respiratory quotient approach very closely to unity (line J, Table III). The freshly defaunated termites also show the same approximate value, and with them no assumption concerning the production of the undetermined gas is necessary. These results confirm the statement of Cleveland that the usual R.Q. of termites is practically 1.0. It is of interest to note, however, that termites which have been defaunated for some time and consequently probably are undergoing starvation show a much lower R.Q.—in the one case here investigated 0.82.[2] This is to be expected from what we know in general of the effect of starvation on the R.Q. of other animals.

To determine the anaërobic carbon dioxide production the same termites were subjected to as low an oxygen tension as could readily be obtained, 0.7 per cent. Since it was ascertained in Experiment 5 that the consumption of this oxygen ceases or is too small to have material effect in about three hours, the termites were allowed to remain for this period in the closed vessels and then the gas exchange was measured for one hour. The readings showed a low but distinct positive variation, indicating the production of gas (line K, Table III). With normal animals part of the output (20 cu. mm./gm. $\times$ hr.) was due to production of the undetermined gas and therefore this amount was subtracted from the observed gas production to give the carbon dioxide value (line L, Table III). This value is considerably below that obtained under aërobic conditions but is evidence that some metabolic changes are still proceeding in the animal's tissues.

To summarize this experiment and answer question 1, it may be said that both normal and freshly defaunated termites produce carbon dioxide under aërobic and anaërobic conditions; that the R.Q. of both types of animal is very close to unity; and that the anaërobic carbon dioxide production while present is much smaller in both types than the aërobic production.

Experiment 7.—Three groups containing equal numbers of termites at 20° C. were placed in air and their respiration measured with and without the presence of KOH. Group I was then exposed to 0.7 per cent oxygen in nitrogen in the presence of KOH for an hour and a half, or until the oxygen was nearly exhausted, at which time air was reintroduced. The oxygen consumption began again and after a brief interval at a lower rate proceeded indefinitely at the same rate as at the beginning.

[2] All the termites were normally mobile and active, even those which had been starved. All were therefore at the same "basal" level.

The absence of any increase in the rate above the normal precludes the possibility of oxygen debt. Groups II and III were similarly treated except that KOH was not present. Air was readmitted at the end of three hours of anaërobiosis. The respiration rates (both carbon dioxide and oxygen) returned very quickly to their normal values and remained there for several hours, at the end of which time the experiment was discontinued.

Experiment 8.—A repetition of Experiment 7 in which the results of the latter were confirmed.

It seems clear that the termite (*Termopsis* at any rate) possesses a mechanism for the continued production of carbon dioxide even in

TABLE IV

The Effect of Prolonged Exposure to Nitrogen

Termite group	Normal oxygen consumption	Hours exposure to nitrogen	Oxygen consumption after exposure	Condition after recovery
	per cent		*per cent of normal*	
I	100	1	89	Immediate recovery from immobility
II	100	3	99	Same
III	100	6	86	Recovery in 15 minutes
IV	100	9	95	Recovery in 20 to 30 minutes
V	100	24	99	Recovery in 1 hour
VI	100	48	96	Recovery in 12 hours
VII	100	96	42	Never recovered
VIII	100	144	16	Never recovered

the complete absence of oxygen. That this mechanism is not identical with that which presumably obtains in mammalian muscle and elsewhere is demonstrated by the complete lack of any indication, from gas measurements at least, of oxygen debt. That the anaërobic system is rather inefficient is shown by the fact that the carbon dioxide production under such conditions is less than one-half the normal value and furthermore by the fact that the termites pass into a state of complete immobility even though they continue to respire. This state resembles acute anoxemia in mammals in that it appears very soon after a sudden exclusion of oxygen and disappears very quickly after readmission of oxygen, a matter of minutes in both cases.

It is worth while to determine how long *Termopsis* can endure complete anoxemia and still retain its capacity for normal aërobic respiration and normal activity.

Experiment 9.—Eight groups of termites, after their normal oxygen consumption was measured, were placed in a desiccator. The latter was filled with nitrogen from which the oxygen had been removed by treatment with strongly alkaline pyrogallol. At intervals the termites were removed and their respiration measured as well as their general behavior observed. The respiratory rate was measured after the termites had recovered their mobility, except in Groups VII and VIII which never recovered. The lack of mobility may therefore in part account for the low rate of oxygen consumption noted in these two groups. The effect of prolonged exposure to pure nitrogen is summarized in Table IV.

It will be observed that the first noteworthy reduction in respiration occurs in the group which had been without oxygen for four days and that this group never recovered their mobility. These termites therefore are not truly anaërobic in the same sense as, for example, yeast, which is able to survive and grow indefinitely in the absence of oxygen. Nevertheless the survival without apparent harm for two days is in itself a striking and significant phenomenon.

In addition to the ability to withstand oxygen lack, *Termopsis* shows high tolerance to the presence of carbon dioxide. The respiration of four groups of termites was measured in 20 per cent oxygen plus 5, 10, 20, and 40 per cent carbon dioxide respectively. The net gas exchange of those in the 5, 10, and 20 per cent carbon dioxide, with no alkali present in the vessels, was approximately the same as that of termites in air and the appearance of the termites otherwise was perfectly normal. It is unlikely therefore that any considerable upset occurred in their respiration. The same considerations concerning respiration apply to the group in 40 per cent carbon dioxide, but these termites soon became immobile.

The general conclusion may be drawn from all these experiments that *Termopsis nevadensis* possesses powers for meeting adverse environmental conditions far in excess of its probable needs. The natural habitat is relatively well aërated wood in which the gas tensions are probably not far from atmospheric. Nevertheless, these animals are capable of extracting practically the last traces of oxygen in a closed space and then of persisting several hours, if not days, in the absence of oxygen. Furthermore, they can endure quantities of carbon dioxide which would seldom if ever be present naturally in their environment. These conditions might occur in wet soil or in other habitats frequented by some of the other species and genera of termites. If this is so, it is possible that ability to withstand such conditions is a general characteristic of all the members of the group which persists in certain

species even though the actual need is seldom encountered. A comparative study of the different species of termites with respect to their respiratory power and their environment is very desirable.

Summary

The oxygen consumption of *Termopsis nevadensis* does not decrease materially with falling oxygen tension until a concentration of approximately two per cent is reached. Below this tension the affinity of the animals for oxygen is so marked that substantially all the available gas is consumed.

In the absence of oxygen the organism is able to respire anaërobically, although at a reduced rate, for as long as two days without injury. During this time the animals are in a state of immobility from which they recover soon after readmission of air. After exposure to anaërobic conditions no indication of oxygen debt was found.

These termites are able to exist and respire normally in carbon dioxide as high as 20 per cent. Higher concentrations tend to induce a condition of anæsthesia, which, however, is reversible.

Under anaërobic conditions, and possibly also in the presence of oxygen, the termites evolve an undetermined gas which may be hydrogen or methane. The production of this gas depends on the integrity of the intestinal fauna, since it is not evolved by defaunated termites.

The respiratory quotient of both normal and freshly defaunated termites is approximately unity, but in starved defaunated termites it falls considerably.

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FURTHER STUDIES OF THE AGGREGATING BEHAVIOR OF AMEIURUS MELAS[1]

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The investigations here reported are continuations of work on the rôle of the sense organs in aggregations of young catfishes (Bowen, 1931), and were directed toward securing further evidence concerning (*a*) the analysis of the factor concerned in the touch reaction; (*b*) the retention of touch and visual aggregating reflexes during prolonged isolation; and (*c*) the influence of aggregation and of isolation upon the physiological processes indicated by the rate of oxygen consumption. Taken as a whole, these studies together with those previously reported contribute to our understanding of the mechanisms concerned in the schooling behavior of *Ameiurus melas* in particular and to some extent to that of fishes in general.

SENSE ORGANS INVOLVED IN THE TOUCH REACTION

The reactions of aggregating young catfishes of the species *Ameiurus melas* have already been studied to determine which sense organs are responsible for receiving the stimuli that result in the formation of the aggregations (Bowen, 1931). Vision was found to be essential for the reactions concerned since blinded fishes did not aggregate and normal fishes failed to aggregate in the dark. There were indications, however, that responses to touch are also of great importance, and that the resulting stimulus may perhaps be the fundamental cause of the aggregations and that vision serves only as a means by which the fishes find one another. Individuals of this species are strongly thigmotactic; they always rest with as many points of the body as possible in contact, and in the aggregations they push against each other continually when disturbed. The components of this contact stimulus were not determined, but the early work indicated that it might contain both tactile and gustatory elements. Morphologically this is possible since (Herrick, 1902) the skin of catfishes over the whole body contains not only end organs which are sensitive to pressure but also terminal buds,

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corresponding to taste buds in the mouth. These are most abundant on the barblets and diminish in number toward the tail. They are sensitive to chemical stimuli through contact as contrasted with the olfactory organs, which are distance receptors (Parker, 1910).

Observations of the behavior of catfishes in the tanks was also suggestive of a gustatory response. One catfish would often approach another, drag its barblets over it and push against it several times. This occurred to some extent when the second catfish was entirely inactive or when an active individual came in contact with a recently pithed fish. The same positive reaction was also given to goldfishes and mudminnows which were in the same aquarium with the catfishes. The response was negative to a weighted model, made of paraffin mixed with India ink to simulate a small catfish in color and form. The approach was the same as to another catfish, but the barblets were drawn over the model only once and then the fish turned away and swam off.

It was considered desirable to analyze further the contact stimulus which one catfish receives from another, and to determine the relative importance of pressure and of chemical stimulation.

An experiment was designed to determine whether there is a difference in the reactions of a catfish to a scoured stone which presumably gives no chemical stimulus and to another catfish rendered incapable of movement and reciprocal pressure. Fishes were pithed and the wound was covered with melted paraffin to prevent the diffusion of body fluids and a resulting gustatory stimulation. Each of these freshly killed fishes was placed at the side of a crystallizing dish 24 cm. in diameter, and held down by a small pebble since the lifeless bodies floated to the top if unattached. A small stone about the size of a catfish was placed at the opposite side of each dish after which a normal individual about 3 to 5 cm. long was added. Observations of the positions of the catfishes were made at 5-minute intervals, or in a few cases after a longer period; after each observation the normal fish was disturbed so that the position in the next observation was determined anew. After five series of records had been made the pithed fishes and the stones were interchanged so that a positive reaction to one place due to light or some other factor would not mask the results. In most cases the active fishes had come to rest at the end of 5 minutes, although in a few instances one or more were still active. If a fish was moving within a small area the location was noted; otherwise these cases were not included in the results.

The results are given in Table I(a). It is readily seen that the number of positive reactions to the pithed fish and to the stone are about the same, 24 and 29 respectively, whereas the total number of indifferent

reactions where the fish was resting apart from either one totals 94. This indicates that there is no difference between the reactions to a stone and to a catfish if the latter is rendered inactive. Immediately upon the conclusion of the first series a control was run by removing the pithed fishes from each dish and substituting a normal fish. These results are shown in Table I(*b*). In 62 per cent of the cases the fishes were resting together as contrasted with 16.3 per cent in Table I(*a*). Since in these cases a response was recorded for each fish, this is equivalent to 31 per cent positive contacts per fish. The increase over the incidence (16.3 per cent) of positive contacts for fishes isolated with one pithed fish is due to stimulation of a visual nature from the moving partner as well as to reciprocal pushing after the two have met. Even with two stones present in the dish with two fishes (Table I, *c*), the

TABLE I

Reactions of catfishes to stones and to other catfishes as shown by resting positions

Condition of experiment	No. of fishes	Positive reactions		Indifferent	Total
		Fish	Stone		
a. Normal fish with stone and pithed fish	5	10	10	27	47
	5	4	6	40	50
	5	10	13	27	50
Total	15	24	29	94	147
b. Two normal fishes with stone	10	62	18	20	100
c. Two normal fishes with two stones	20	98	48	54	200

fishes aggregated about as much as in the preceding arrangement where the single stone allowed less possibility of purely indiscriminate contact. In about half of these observations the two fishes were found resting together, an equivalent of 24.5 per cent positive contacts per fish, and a decided increase over the 16.3 per cent in (*a*) where an inert partner was present.

Since it had been found that catfishes responded negatively to a black paraffin model, it was possible that the small amount of paraffin upon the pithed fish might be preventing a positive reaction to the pithed fish itself. Accordingly the responses to a paraffined and to a non-paraffined stone were compared. An equal number of positive reactions, 24, was given to each object with 88 indifferent responses, so

that it may be concluded that there is no definite reaction to the paraffin itself.

From the above results there is no evidence for a gustatory element in the stimulus which one catfish receives from another. When movement and pressure are eliminated the positive response is given in as many cases to a stone as to a catfish. The gustatory stimulus enters only to produce a negative reaction when some unfavorable chemical is present. Observations upon normal fishes in a dish with untreated paraffin models and similar models mixed with India ink to add color showed that a decidedly negative reaction was given only to the India ink models. To untreated paraffin models the reactions were the same as to an inactive fish. It was impossible to eliminate the sight reflex by using blinded fishes in these experiments, because a blinded fish remains active for long periods without coming to rest near any object; such fishes rest only after long activity and in the absence of recent stimulation.

Effect of Isolation upon Aggregating Behavior

The question as to whether the reactions concerned in catfish aggregations are entirely instinctive and automatic or are in part due to conditioning or at least susceptible to modification by conditioning is of interest in this simple type of social behavior found low in the vertebrate scale. Parr (1927) explained the schooling of pelagic fishes by an assumption of a simple automatic eye reflex which acts in the case of milling to produce a type of behavior with no apparent purpose. Learning among fishes has, however, repeatedly been demonstrated. Thus individuals have been trained to associate a stimulus such as light, color (Hineline, 1927), or direction (Churchill, 1916), with food, or in Goldsmith's work (1914) with the nest; and Triplett, 1901, by separating a perch from minnows by a glass partition, conditioned the perch so that after the removal of the partition it made no attempt to reach the minnows by passing the line where the partition had been. Recently Bull (1929) has shown that *Blennius pholis* can be trained to use the senses of taste and smell in the capture of food, although normally they play no part in this behavior. He has demonstrated that purposive movement in fishes can be explained by a combination of unconditioned and conditioned responses in nature as well as in the laboratory.

Two types of reactions of catfishes to one another had previously been observed (Bowen, 1931). Normally vision is the means by which individuals are enabled to come in contact with one another. In addition blinded fishes, previously grouped, were shown to respond to each other when the skin was intact by turning out of their course toward a passing fish in approximately 50 per cent of the cases where the

two fishes came within two inches of each other. This reaction is apparently due to vibrations set up by the tail of the passing fish and received by the skin, and which act possibly more or less as a thigmotactic stimulus. This positive response may result only after fishes are blinded, but it serves to bring the fishes momentarily nearer one another, and may therefore be considered as a social response.

If the satisfaction of a thigmotactic response is the fundamental basis of aggregating behavior in catfishes, then it seems possible that both the above types of response might be modified if, through isolation, thigmotactic responses from other fishes were prevented for a time. The first indication that such might be the case was found in work with gradient tanks (Bowen, 1931) in which blinded catfishes, isolated from the group for a week, showed a tendency to stay in the opposite end of the tank from the group of fishes when the latter were separated from the single individual by a wide-meshed wire partition. The observed difference in behavior was statistically significant; at the same time blinded fishes which had been members of a group tended to stay in the end toward the group, although the difference from the controls was not significant with blinded fishes.

To determine whether the response of blinded fishes could be conditioned by isolation, individuals were placed in two-quart jars containing plants and fed regularly for a period of 20 days. They were then placed in groups of five in a porcelain tub and the reactions recorded for 15 minutes. At the conclusion of these observations they were kept grouped for one or more days and tested again as a control to determine whether they gave the normal number (50 per cent) of positive reactions after grouping. In almost all cases a positive reaction was given when two fishes actually touched. The few exceptions occurred when they met with some force and darted back in a negative response. In cases where the fishes passed within two inches of each other without touching, however, a difference between previously isolated and grouped fishes was evident. While the latter gave a total of 27 positive responses, 1 negative and 26 indifferent, showing 50 per cent positive reactions, isolated individuals gave 14 positive, no negative, and 38 indifferent responses with only 27 per cent positive. The difference is, however, actually even greater between the two groups; during the first 9 minutes after grouping of previously isolated fishes there was only one positive response and 30 indifferent ones. During this period there were several positive reactions to contact, which apparently served to overcome the effects of isolation, so that the positive reaction was reëstablished at the end of that time. In fact the positive responses for the next 6 minutes rose to 62 per cent of the total.

From this series it seems evident that the positive responses of blinded fishes to one another are eliminated by isolation, but are re-established again in a few minutes when the fishes are in the same container and contact may occur. The ease with which the response of blinded fishes to vibrations caused by another fish is broken down and the fact that several minutes are required for its reëstablishment make it seem possible that this is a true conditioned response developed after the fishes are blinded, and in connection with the thigmotactic stimulus. It does not act with enough efficiency to enable the formation of aggregations, as one fish can determine the direction of another fish for only one or two turns and then loses it. In normal individuals vision directs the movements of the fishes toward one another at a greater distance than this stimulus can be felt so that it probably acts very slightly if at all in aggregations of normal fishes.

The effect of isolation upon the aggregating behavior of normal fishes was also tested by a similar series of experiments. Several attempts were made to isolate very young catfishes to determine how readily the normal response might be altered before the characteristic social reactions had become established by long use. But it was impossible to keep the very young fishes alive in the laboratory. They died in large numbers under the best conditions and none survived a spell of hot weather. Therefore most of these results were obtained upon older fishes.

The method employed in recording the reactions of the fishes to one another was similar to that for the blinded fishes except that a distance of about a foot and a half was considered the maximum within which a positive reaction was possible, since normal fishes nearly always react to one another within this distance. In all cases the two or three fishes placed in the experimental tub at one time were aggregating closely in the course of a few minutes, so that reactions were recorded only until this occurred. These were of two types, touch reactions where two fishes came in contact without any observable turning from their course when within visual distance, and sight responses where one of the fishes turned definitely toward the other. When either resulted in typical aggregating behavior it was considered positive; if not, indifferent. Usually a touch stimulus resulted in a positive response but in a few cases, almost always with previously isolated fishes, the first touch response was indifferent, but was followed shortly by a positive response often quite clearly as a result of a sight stimulus.

The results of all the experiments are given in Table II. The effect of isolation upon reactions responsible for aggregations seems to be shown most clearly by the number of indifferent reactions as compared

with the positive sight reactions before aggregation is established. In each case where there were indications of a breakdown of the aggregating behavior, the fishes were kept together for a day and the experiment repeated as a control. Such procedure was necessary for comparison since normal fishes which have been isolated for a long period of time require several days before they become as active or responsive as those from a group, a condition which might affect the proportion of positive reactions.

The isolation of very young fishes for five days (Experiment 1, Table II) gave an indication of a breakdown of the aggregating re-

TABLE II

Summary of reactions of catfishes to one another after isolation

Exp.	Number of fish	Number tested together	Length	Days isolated	Indifferent reactions before positive response			Positive sight reactions before contact
					Sight	Contact	Total	
1.	6	2	1 in.	5	6	1	7	1
1(*a*).*	6	2	1 in.	0	3	0	3	0
2.	8	2	3–5 in.	38	0	1	1	3
3.	8	2	1.5 in.	52	0	0	0	6
4.	3	3	2 in.	161	4	0	4	0
4(*a*).* (from Exp. 3)	3	3	2 in.	0	0	1	1	1
5.	12	3	2 in.	161	5	5	10	2
5(*a*).*	12	3	2 in.	0	1	1	2	5

* In (*a*) of each experiment are the results when the fish were retested after one or more days together.

sponses. In Experiments 2 and 3, where young fishes about six months of age were isolated for between one and two months, the behavior was not modified and was entirely typical of fishes from the group. In Experiments 4 and 5, however, where the individuals were kept isolated for over five months, there was decided evidence for an isolation effect. Under these conditions the fishes isolated for five months gave in all 9 indifferent sight responses and 5 indifferent touch responses as compared with one of each after they had been together for 24 hours. Only 2 positive sight responses were noted before contact occurred as contrasted with 6 in the control. As the sight stimulus still acted in two

cases without a touch stimulus, the former was not completely eliminated, but there seemed to be a decided tendency in that direction.

Whether this sight reflex is instinctive or whether it is established soon after hatching as a conditioned response depending upon the positive thigmotactic reaction is still a question. If instinctive, however, it is apparently subject to change by the conditioning processes involved in isolation if these act for a long enough period of time. Probably also this effect can be produced more readily the younger the fishes. Unfortunately observations on the initial reactions of newly hatched fishes could not be made, hence the obvious and crucial test for the relative amounts of instinctive and conditioned elements in this behavior complex is lacking. However, the evidence indicates that conditioning plays a part in this aggregating behavior.

From a survey of the results obtained to date in the investigation of the reactions responsible for the aggregations of young catfishes, we may conclude that the two senses concerned are sight and contact with taste playing no part in spite of the acuteness of this sense in these animals. Sight is the sense by means of which the fishes normally aggregate and the visual response of catfishes may be an instinctive reaction to one another. Touch, however, probably has the more basic rôle, since the sight response can be diminished somewhat in effectiveness by isolation and is reëstablished by contact. The mutual pressure of the fishes due to pushing seems to give the stimulus which maintains these aggregations.

Respiratory Behavior of Grouped and Isolated Catfishes

It has been shown that grouping has an effect upon the respiratory rate of animals. Thus Allee (1926 and 1927) has found that in land isopods and the brittle starfish, *Ophioderma brevispina,* at least when out of the breeding season, the rate of respiration, as measured by the amount of oxygen consumed, is lower per animal for grouped individuals than for isolated ones for the first few hours. The rate of respiration decreases more rapidly for the isolated animals, however, so that after several hours these are respiring more slowly than the grouped ones. There is evidence that, in the case of the starfishes at least, the opportunity for physical contact afforded by the other individuals present is responsible for the group effects upon respiration and for the longer survival of the individuals composing it. Schuett (1931) has investigated this phase of respiratory behavior among goldfishes, guppies, mudminnows and *Fundulus heteroclitus,* and has obtained results similar

to those of Allee for land isopods and brittle starfishes. In his shorter experiments, which covered usually one to five hours, the group consumed less oxygen per fish than did the isolated individuals. Working independently, I have been able to confirm Schuett's results with goldfishes, the only one of his species tested.

It seemed desirable to determine whether any difference exists between the respiratory rate of isolated and grouped individuals of aggregating young catfishes, especially in view of the part that contact plays in such reactions. Accordingly experiments were run with both normal catfishes and with catfishes which had been blinded several days previously in order to eliminate the important effect of vision upon aggregating behavior.

The method followed the technique of Schuett. Each experiment was performed in parallel with normal and blinded fishes. In each case, four single fishes were placed in individual Erlenmeyer flasks, holding one liter, and four were placed together in a similar flask. The flasks were then arranged parallel to two windows to equalize the normal effect of light upon the behavior of catfishes. The flasks had previously been filled with well water which in most cases had been allowed to stand overnight to come approximately to room temperature and to saturation with air, although in the first experiments air was bubbled through the water. In all of the experiments the oxygen tension of the initial samples varied only between 4.5 and 6.5 cc. per liter of water; in this range the rate of oxygen consumption is independent of the oxygen tension (Schuett and citations). After the introduction of the fish, a layer of heavy mineral oil was poured into the neck of the flasks to prevent gaseous exchange at the surface. By means of glass siphoning tubes kept in the flasks, samples of about 15 cc., known to within 0.05 cc., were withdrawn for analysis for oxygen by the method of Winkler. One sample was taken immediately after the introduction of the animal and a second one one hour later. A control flask without fish was sampled similarly. The external end of the siphon terminated in a piece of rubber tubing closed by a clamp. In the first half of the experiments the siphons reached to the middle of the flasks. In the last ten experiments longer siphons were employed and the water was stirred with the siphon one minute before sampling. No difference was noted in the results with the later modification of the technique. The fishes to be used in these experiments were kept on the experimental table in glass-walled aquaria to avoid excessive stimulation while being transferred to the flasks.

Table III shows the results of the experiments. The total oxygen

consumed by four isolated fishes in each experiment is compared with that consumed by the group. The difference which Schuett and I have found between grouped and isolated non-schooling fishes is not obtained here. In fact, with the normal catfishes the members of groups consumed on the whole more oxygen than did isolated individuals but

TABLE III

Total oxygen consumption for one hour of four grouped and four isolated catfishes, blinded and normal (expressed in cc. oxygen per liter)

	Normal		Blinded	
	Isolated	Group	Isolated	Group
1			.56	.20
2	.79	.81	.40	.57
3			.41	.34
4	1.16	1.03	.80	.63
5	.47	.67	.95	.49
6	.64	.64	.42	.49
7	.62	.66	.67	.51
8	.65	.68	.68	.68
9	.26	.42	.73	1.03
10	1.02	.98	1.12	1.27
11	.80	.74	1.00	1.01
12	.72	1.11	1.29	1.02
13	.40	.70	.56	.72
14	.88	.48	.75	.50
15	.31	.54	.48	.42
16	.88	.55	.93	.51
17	.34	.61	.23	.61
18	.77	.55	.59	.39
19	.39	.50	.54	.36
20	.87	.88	.72	.88
21	.63	.63		
22	.46	.57	.59	.53
23	.48	.45	.69	.86
24	.20	.45	.55	.53
25	.18	.41	.46	.42
	Mean difference 0.05087 cc. more oxygen for groups. Probability 0.2158.		Mean difference 0.05 cc. more oxygen for isolated fishes. Probability 0.2898.	

the difference is not statistically significant. In the case of the blinded fishes the isolated individuals consumed very little more oxygen, but here again the difference is not important. The results of three typical experiments are given in Table IV.

The cause of the discrepancy from Schuett's and my results with goldfishes is not difficult to find, nor do these indifferent results offer

any contradiction to his. With normal catfishes the isolated individuals remain quiet during practically the whole experiment. In the groups, on the other hand, the fishes are in motion most of the time, being stimulated by the presence of the other individuals. Thus the rate of respiration of the grouped fishes is increased and the total is higher than for the isolated fishes. The fact that the difference is no greater probably indicates that if the factor of movement could be eliminated with cat-

TABLE IV

Results of three experiments, showing oxygen consumption of isolated and grouped fishes, blinded and normal

Experiment	Normal		Blinded		Control
	Isolated	Group	Isolated	Group	
18	.23		.19		
	.09		.16		
	.18		.07		
	.27		.17		
	.77	.55	.59	.39	.04
19	.10		.07		
	.12		.13		
	.14		.15		
	.03		.19		
	.39	.50	.54	.36	−.04
20	.14		.28		
	.13		.03		
	.34		.18		
	.26		.23		
	.87	.88	.72	.88	−.06

fishes a lower rate of respiration for the groups would be found than for the singles, in agreement with Schuett's work on goldfishes, where problems connected with relative motion were apparently not involved. With the blinded fishes both isolated and grouped individuals are in motion all the time, as are goldfishes, but more actively so. Here the difference is in the same direction as in the case of goldfishes, but is less and not as consistently positive. This is not surprising with the amount of motion occurring with these blinded fishes.

It was hoped that contact responses which gave as much satisfaction as they apparently do in catfishes might produce a difference in the respir-

atory rate, and that this might serve as a method of determining more accurately the rôle of the different sense organs in the social behavior of these fishes. So far this has not been possible, but the results do indicate an interesting difference between the respiratory rate of schooling and non-schooling fishes; at least in these schooling fishes, such as *Ameiurus melas,* individuals stimulate one another to activity by sight or touch and thus offset or counteract the lower respiratory rate found among groups of non-schooling fishes, so that there is no significant difference in oxygen consumption between grouped and isolated individuals. With blinded fishes it may be that contact stimulation is effective in raising slightly the respiratory rate of the group so that here a significantly lower rate was not obtained.

Summary

Catfishes showed no discrimination by contact between a scoured stone and an inactive catfish, nor between a paraffined and non-paraffined stone, but gave a negative reaction to models of paraffin mixed with India ink.

There is no evidence for a gustatory element in the stimulus which one catfish receives from another. A gustatory stimulus acts only to produce a negative reaction when some unfavorable chemical is present.

The positive responses which blinded catfishes give to one another in passing are eliminated by isolation for 20 days, but reëstablished in a few minutes when the fishes are placed together.

Touch responses by blinded catfishes are positive immediately after isolation when contact is gentle enough to prevent shock.

The reactions of blinded fishes to one another due to a response to water vibrations, may be a conditioned response developed after the loss of eyesight, and is probably not effective among normal fishes.

The sight response of normal fishes to one another was not completely eliminated in all individuals by 161 days of isolation, although it was much less marked. It was reëstablished in the course of a few minutes, usually soon after contact occurred. This sight response may be instinctive but is probably subject to modification by conditioning to some extent at least.

A satisfaction evidently accrues to the catfishes from the mutual contact and pressure of the aggregations, and the importance of the thigmotactic response in these reactions is emphasized by these observations.

A comparison of the respiratory rate of catfishes, both normal and blinded, for a period of one hour gave no significant difference between the grouped and isolated individuals.

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MECHANISM OF MOVEMENT OF EPIDERMIS, ESPECIALLY ITS MELANOPHORES, IN WOUND HEALING, AND BEHAVIOR OF SKIN GRAFTS IN FROG TADPOLES

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Probably no other animal tissue is more active than an epidermis in repairing lost or damaged parts. The process is not primarily one of growth. A deficiency, either large or small, in the epidermis is rapidly covered by centripetal movement of the surrounding epithelium, and later, by reorganization and growth, the original condition is restored. Rand (1915) in considering the wound reactions of actinians says (p. 207): "In general, an epithelium will not tolerate a free edge. When such an edge arises, accidentally or otherwise, the epithelium extends until, if possible, the free edge meets and unites with some other portion of the same layer or with another epithelium. The essential function of an epithelium is to cover a surface continuously."

The investigations of Fraisse (1885), Barfurth (1891), Born (1896), Morgan (1901), Loeb and Strong (1904), Rand (1905), Eycleshymer (1907), Matsumoto (1918), Loeb (1920), Arey (1925), and Collins and Adolph (1926) point unanimously to the conclusion that wounds are at first covered by movement of the surrounding epithelial cells and that proliferation occurs later to restore the original thickness of the layer.

The cause and mechanism of the cell movement are not so well agreed upon. Born (1896) believed that the cells flatten to cover a larger surface than previously. Rand (1905), however, referring to wound healing in earthworms, says (p. 46): "There certainly is little evidence in favor of supposing that the concentric advance of the epidermis is due to the tendency of the individual cell to spread itself over the greatest possible surface."

Barfurth (1891) proposed that, while the movement of the epidermis might be in part a passive "Verschiebung" due to relief from lateral pressure in the layer, it was in the main an active movement of cells which had become "embryonal beweglich (amöboid)" (p. 417). Oppel (1912) described epithelial movement as an active movement—often a "Massenbewegung"—resulting from change of form of the

epithelial cells which, however, are not ameboid. On the contrary, Holmes (1914), observing amphibian epidermis in tissue culture, decided that epithelial movement is not a "Massenbewegung" but is the result of essentially ameboid movements of individual cells. And, again, Collins and Adolph (1926) concluded that wound closure is accomplished by a mass movement or "pushing in" process of the epithelial layer and "not by the independent migration of cells" (p. 491). Loeb and Strong (1904, p. 282) considered it probable that "tension, either previously existing or called into play by the wound, is the cause" of the closure of the epidermal wounds.

Morgan (1901, p. 70), describing wounds in very young tadpoles, stated that "the wound is covered not by individual cells wandering over the exposed surface, but by a steady advance of the smooth edge of the ectoderm toward a central point. . . . As there are no muscle fibres present . . . , the result cannot be due to muscular contraction. . . ."

The artificial cultivation of epithelial tissue has given striking evidence that the cells are motile and their movement ameboid. This was suggested at an early date by Peters (1889), who studied wound closure in the cornea of frog eyes. Tissue culture studies have been carried out by Loeb (1902 and 1912), Harrison (1910 and 1914), Holmes (1913 and 1914), Oppel (1913), Uhlenhuth (1914 and 1915), Hooker (1914), Matsumoto (1918), Maximow (1925), and others. For the most part these investigators agree that movement of epithelial cells over a wound surface is ameboid and usually stereotropic.

The cause initiating the cell movement is not definitely known. Rand (1905) suggested a wound stimulus: "The most important factor in the earlier part of the process of reparation is cytotaxis; the individual columnar cells of the existing epidermis are affected by some directive stimulus and respond by an active migration, which results in the covering of the surface of injury by a protective epithelium—the first definitive step toward regeneration" (p. 52). Taube (1923) concluded that materials of injured cells flow over wound surfaces and serve as a means of chemical stimulation for the uninjured cells at the edge of the wound, causing them to migrate and later to divide. Maximow (1925) suggested that regenerative proliferation may be the result of direct stimulation by the injury or of the action of specific chemical substances. The trend of recent conclusions is in the direction of the activation of epithelial cells by a wound hormone or wound stimulus of some sort.

In the following pages are reported studies upon the skin of tadpoles of *Rana clamitans*. The studies were carried out with a view to

further understanding the movement of epidermal cells over a denuded area, the behavior of host tissue in relation to a skin graft, and especially the mechanics of radial arrangement of epidermal pigment cells around a wound.

The animals used in these experiments were collected from ponds or streams near Cambridge, Massachusetts. They were usually 70 to 80 mm. in length.

The Mechanics of Wound Closure

It has been frequently noted that epidermal wounds close very quickly. In tadpoles, skin wounds 5 to 10 mm. across may be entirely

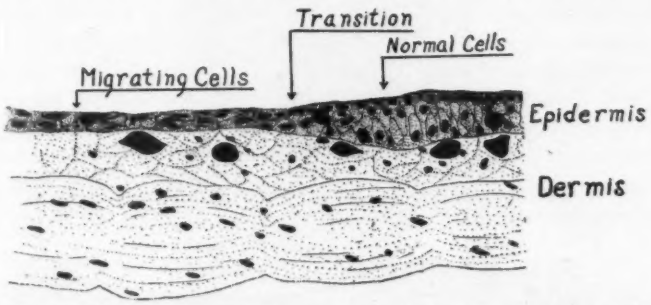

FIG. 1. Section perpendicular to the surface of skin near a wound, showing migrating cells and the transition to those not migrating. The wound was a short distance to the left of the cells illustrated.

closed in from 6 to 24 hours. Upon the occurrence of a wound, a quantity of blood always flows into the area and there coagulates. The coagulum usually nearly fills the wound temporarily, being about equal in thickness to the skin. Continuous microscopical examination, immediately following the operation, revealed that the edge of the epidermis starts to move toward the center of the wound within a few minutes after the wound is made. The epidermal layer advances over the coagulum, moving equally from all sides toward the center of the wound. If the edge of the wound is irregular, the advancing layer is correspondingly irregular in outline. Over large wounds the advancing epidermis thins out until it may be only one or two cells in thickness. The thinning may extend several millimeters distant from the wound, depending on the size of the wound. Figure 1 shows the limit of the thinned region of the layer and the transition to the normal condition which in this case is about 5 mm. from the edge of the rather large wound. The advancing cells are quite undifferentiated. Those of the

cuticular layer are hardly distinguishable from those of other layers of the epidermis.

Sections parallel to the surface of the layer were cut from normal epidermis (Fig. 2) and from areas adjacent to a closing wound (Fig. 3). In such sections the normal cells exhibited fairly regular polygonal

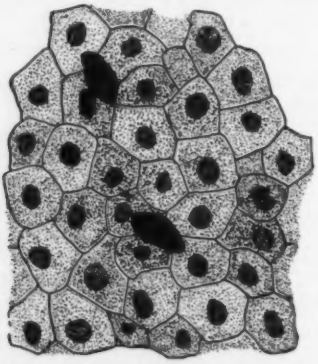

FIG. 2. Section parallel to the surface of normal epidermis. The solid black areas represent unexpanded, epidermal melanophores.

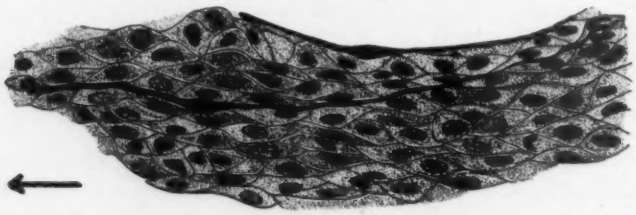

FIG. 3. Section parallel to the surface of epidermis near a wound, showing cells elongated in the direction of movement. The cells are moving in the direction of the arrow. The elongate black areas represent expanded, epidermal melanophores.

outlines while in an area near a closing wound the cells were from two to four times as long as wide, with the elongation in the direction of movement. This elongation of cells occurs at all points between the edge of the advancing layer and the region of transition to the undisturbed epidermis. Several writers have suggested that epithelial cells elongate perpendicularly to the edge of a wound, but an extensive reading of the literature discovers no mention of appropriate sections having been made to establish the fact. Sections perpendicular to the surface merely suggest the possibility of elongation.

When advancing edges of epidermis meet at or near the center of

the wound, there often takes place a piling up of cells. This piling up suggests that the causal factor for migration continues to operate for a time after the advancing edges have come together. After the wound has been covered by a layer of epithelium, the coagulum disappears. Within two or three days mitosis begins and the layer over the wound continues to increase until it is somewhat thicker than the epidermis in other parts of the body. Also the deficient epidermis surrounding the wound is built up, by cell proliferation, to the normal thickness of the layer.

In wounds made entirely through the skin the dermis is slow in closing, but the epidermis closes as usual, subsequently thickening until it is much thicker than the normal epidermis. Sometimes the thickness of the layer over a healed wound may be equal to the combined thickness of the normal epidermis and the dermis. When transplantations are made, the small gaps that necessarily exist between host skin and graft are closed by epidermis which grows in to fill the deficiency as if to maintain a smooth surface to the body. As the dermis is built up, the epidermis returns to its usual thickness. The function of epithelium may be not only "to cover a surface continuously," as declared by Rand, but also to restore a smooth external surface after small injuries.

Skin Grafts and the Compatibility of Tissues

The diversely pigmented integument of amphibians, possessing extraordinary capacity for repair and regeneration and readily amenable to grafting operations, offers many obvious advantages for the study of problems in tissue compatibility.

A patch of ventral, unpigmented skin from a tadpole was transplanted to the back of the same animal. Twenty-six autotransplants of this kind were made. In each instance the transplanted piece was 5 to 7 mm. square. Several operations were performed in which a dorsal patch of skin was merely cut loose and then reimplanted where it had previously been. Other patches were rotated through 90° or 180° and reimplanted.

The epidermis of an autotransplant united with that of the host immediately after the graft was made, but after that union had been effected there was no further movement on the part of the host tissue. In contrast to what happened in homoiotransplants, there was no invasion of the graft by host tissue. There was no sign of incompatibility between the ventral and dorsal skin tissue. Once the epidermal layers had united, the regenerative activity ceased. Dorsal pigmented patches which were rotated or reimplanted in their original positions were not disorganized and in a few weeks the limits of the grafts could not be

recognized. The half-white, half-pigmented, lateral patches which were rotated through 180° remained unchanged, the light area of the patch remaining unpigmented and the pigmented end retaining its pigment with none spreading into the surrounding white area (Fig. 4). Animals bearing this type of graft were observed for as long as 80 days. Collins and Adolph (1926), however, observed disorganization of rotated skin grafts in experiments on *Triturus*. It seems clear that no incompatibility exists between dorsal and ventral skin of an individual tadpole, although, in respect of pigment, they are locally specific.

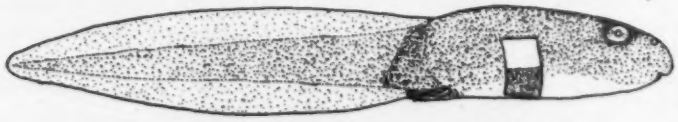

FIG. 4. Frog tadpole showing lateral, rotated graft.

Homoiotransplants were made by grafting ventral unpigmented skin from one animal onto the back of another. The grafts healed in place very rapidly so that in a few hours a microscopic inspection of the tissue in the region of the union would scarcely reveal just which were host and which were graft cells. Not all of the movement, however, in covering such gaps is accomplished by the host epidermis, for that of the patch moves almost as rapidly until the two unite. The dermal layers of host and patch are much slower in uniting than the corresponding epidermis, requiring many days or even weeks.

Sixty homoiotransplants were observed. Without exception they became occupied by host epidermal cells; and, if the animals lived long enough, dermal cells entered the unpigmented area. The grafts began to become pigmented in from 1 to 24 days after transplantation, the average being 9. The host epidermis, carrying the epidermal pigment cells, moved centripetally into the graft quite equally from all sides. Once having started, an average of 6 days elapsed before the pigment-bearing host epidermis reached the center of the patch. From a cursory examination of the surface, it was believed that the host epidermis grew over the patch, covering the epidermis as a whole. A study of microscopic sections, however, gave no indication of overgrowth or undergrowth of the epidermis, in accord with the account given by Cole (1922). The epidermis of the patch evidently undergoes destruction and is simultaneously replaced by that of the host. What appeared to be the remains of disintegrated graft cells were found in the cells at the edge of the advancing host epidermis, suggesting phagocytosis. That epidermal cells may be phagocytic at times has been stated by Loeb (1902, 1912).

The invasion of a graft by host epidermis is essentially a process of delayed wound healing. The delay is necessitated by the presence of the graft epidermis. Except for complications occasioned by the removal of the patch epidermis, the behavior of the host skin in relation to the homoiotransplant is nearly like that in ordinary wound closure. The host cells near the patch become elongated in the line of movement just as in wound healing (Fig. 3).

Fourteen transplantations were made in which patches of dorsal skin were transferred to wounds on the ventral surface of the animal. Whether the graft was an auto- or a homoiotransplant, healing took place as usual, but in the course of a few days the patch showed signs of degeneration and subsequently sloughed off until all or the greater

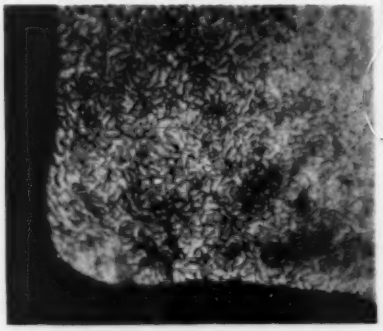

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FIG. 5. Photograph of corner of skin graft showing the network of enlarged blood vessels.

part was lost. It is believed that this condition was not necessarily the result of incompatibility between the tissues but that the ventral side of the body, with its very thin body wall, is an unfavorable place for a graft to receive nourishment.

BEHAVIOR OF BLOOD VESSELS IN SKIN GRAFTS

Nearly all skin transplants early become red because of the enlargement of their blood vessels. The degree of redness varies from slight traces to a deep, solid red covering the entire patch. The reddening begins as blood vessels here and there over the patch become enlarged for very short distances. The enlarged regions then extend until a close network of such vessels may be seen over any portion or all of the patch (Fig. 5). The redness becomes apparent usually the

second day after the graft is made, but it has been observed as early as 18 hours after an operation, with definite circulation in the enlarged vessels at 24 hours. The redness may persist for one to two weeks. In the greater number of cases the blood vessels enlarged until, in a few or many places in each graft, they broke to release blood into the loose dermal tissues, causing the appearance of a continuous layer of blood. This stage persisted usually less than a week. As it cleared, blood vessels of normal size could be seen, suggesting repair of the vessels. Since the vessels that occasioned the temporary redness became filled with blood within the short time of a few hours after the operation and because their arrangement is so similar to that of the vessels normally present in the ventral skin, it is probable that they are not newly developed after transplantation, but are the original vessels of the grafted skin.

Cole (1922) has described the reddening of homoiotransplants of tadpole skin but states that autotransplants do not show this reaction. He says (page 391): "The occurrence of such a process is evidence of a rather violent chemical reaction going on between the protoplasms of the graft and host, and a merely specific difference between the two could set up the reaction." Cole's conclusions, however, are not in accord with the experiments described in the present paper, for in these experiments both auto- and homoiotransplants became reddened in nearly all cases. Since white autotransplants never became pigmented and since there was no other indication of any reaction between tissues, it seems evident that increased vascularity is not an indication of incompatibility between tissues. Furthermore, there is considerable evidence that enlargement of the blood vessels is due to mechanical stretching of their walls. As circulation becomes established in the graft, the blood exerts pressure on the walls of the vessels. In normal tissues, tonus of the vessels balances this internal pressure. There is slight variation in the caliber of normal vessels to meet the varying needs of the animal, the tonus being controlled by the nervous system. In transplanted patches the nervous connections have been cut during the operation, leaving the blood vessels of the patch without normal tonus, only a certain elasticity intrinsic to the vascular tissue itself persisting. It is highly probable, therefore, that enlargement of blood vessels following transplantation is due to stretching of the vessels from internal pressure in the absence of nervous control and not to a specific reaction between tissues as concluded by Cole.

May (1924), after transplanting lizard (*Anolis*) skin, concluded (p. 553): "Transplants of pigmented skin lose their colour-changing

power immediately on being completely disconnected from neighbouring tissues, and regain it slowly as nerves regenerate." It has not been ascertained just when nerves regenerate to innervate the grafted tadpole skin, but the apparent resumption of control of the blood vessels agrees in time with that given by May for the regeneration of nerves in the skin of *Anolis*. May's conclusions apply very well to the apparent loss and regaining of nerve control of the blood vessels of the tadpole grafts.

Radial Arrangement of Epidermal Pigment Cells Around a Wound or Skin Transplantation

When expanded epidermal pigment cells lie upon or near a skin wound which is healing or a transplanted patch of skin which is be-

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Fig. 6. Photograph showing the nearly parallel arrangement of epidermal pigment cells near a wound.

coming pigmented, they show a particular arrangement in relation to the wound or graft. In general, the axes of elongated melanophores are perpendicular to the edge of the wound or, in other words, they are parallel to the line of movement of the epidermal cells toward the wound or graft. In any one localized area the greater number of epidermal melanophores are parallel (Fig. 6). Some cells may be at an angle to others, or occasionally a melanophore may be perpendicular to the axes of the greater number, but such cells are too few to impair the conspicuous radial arrangement of the majority. This arrangement may extend for several millimeters external to the edge of the wound or graft. The area over which radial arrangement may take place was found to coincide with the extent of the region within which migration

of the epidermal cells occurs. In Fig. 1 the limit of radial arrangement occurs at the point labeled " transition." This observation is very significant in relation to the mechanics of the radial arrangement.

It is obvious that radial position of a pigment cell will not be evident unless the cell is expanded. Radial arrangement depends upon expansion of the melanophores, but the causes of radial arrangement and expansion were found to be distinct.

Since investigation of the causes of radial arrangement could be carried on only when the epidermal melanophores were expanded, it was desirable to find an artificial means of producing expansion. The following technique was used. An injection fluid was prepared by adding desiccated bovine pituitary gland to Ringer's solution and filtering the mixture through filter paper. The powdered gland material dissolved and filtered better if the liquid were slightly warm. About one gram of desiccated gland was used in 10 cc. of Ringer's solution, but wide variations of this concentration proved to be effective. Intraperitoneal injections were made, using a small hypodermic syringe with a dosage of about 0.2 cc. After an injection of this kind the melanophores began to expand in about twenty minutes and continued expanding for one or two hours until maximal or nearly maximal expansion was reached. Allen (1917), Swingle (1921), and Collins and Adolph (1926) have studied in considerable detail the effect of pituitary gland and its extracts on pigment cells, but in the experimental work now being described the pituitary extract was used merely to expand melanophores in connection with the study of the problem of their radial arrangement.

Expanded epidermal melanophores near a wound which was being covered, or around grafts toward which epidermis was moving, exhibited more or less marked radial arrangement. Therefore radial arrangement of melanophores may be expected wherever there is a migrating epidermis whose melanophores are expanded. After a wound or graft has been covered by the surrounding epidermis and the layer has nearly returned to its normal thickness, the melanophores begin to lose their special arrangement. In homoiotransplants radial arrangement persisted from one to two weeks or even longer. In one large homoiotransplant, after it had become pigmented and the radial arrangement lost, two holes, about 3 mm. apart and each about 1 mm. across, were cut through the skin of the graft. The pigment cells were partially expanded. Twelve to eighteen hours afterward there was marked radial arrangement around each of the holes.

In considering the mechanism of radial arrangement of melanophores, the conditions of both normal and migrating cells must be kept

in mind (Figs. 2 and 3). During the migration of the epidermal cells, their space relationships are continually changing. Further, *the epidermal cells closest to the edge of the wound or graft move faster than those successively farther from the wound or graft.* In the initial advance of cells in covering the wound, the cells closest to the edge of the wound travel a greater distance than cells successively farther from the edge. It is believed that the mechanism of radial arrangement may be completely described from the facts just stated.

Unless a pigment cell is lying with its long axis exactly perpendicular to the line of movement of the epidermal cells, the end of the melanophore nearer the edge of the wound will be carried faster than the remainder of the melanophore. The carrying of one end of the cell faster than the other in a given direction will tend to rotate the comparatively long pigment cell to lie in a new position, more nearly in the line of movement of the epidermal cells. Since a single epidermal melanophore may be as long as the diameters of twenty epidermal cells, this action could easily occur. Continuous microscopic observations made on living epidermis at the edge of a wound support in every detail the suggestion just made. Such observations were made continuously on a single wound for as long as twelve hours.

Suggestions for apparatus used in making the observations just mentioned were obtained from Clark (1912). A small glass box was constructed, part of one side of which was made of "cover slip" glass to permit the use of high-power microscope objectives. The box was filled with paraffin to a depth of about 5 mm. The tube of an ordinary compound microscope was turned to the horizontal position and the glass box was secured to the mechanical stage. With the box in this position, the open side was uppermost and the side made of the thin glass was next to the objective lens of the microscope.

When a wound was to be studied, the tadpole was first anesthetized by placing it in water to which had been added a few drops of ether. As soon as anesthesia was produced, the animal was placed in the glass box, which contained a 0.05 per cent chloretone solution. Chloretone is a very slow-acting anesthetic and causes melanophores to expand. Ether was used as described merely to hasten immobilization of the animal before the melanophores could expand. The animal was held against the thin side of the box by means of pins thrust into the paraffin.

Usually transmitted light from the mirror or directly from a lamp was used, but reflected light with or without transmitted light was sometimes used. Sufficient light could be transmitted through the tail of a tadpole to permit the use of an oil-immersion lens, but the best results

were obtained by means of a 4 mm. lens and a 10 × to 18 × ocular. Magnifications up to nearly 1000 diameters were frequently obtained. The animals remained alive in the glass box for over 24 hours, so that very satisfactory records were obtained up to that length of time.

A vertical drawing board was used to permit the employment of a camera lucida. Individual pigment cells were located and followed for several hours. Hence by making drawings at frequent intervals, the exact courses of the cells could be followed. The movement was too slow to be observed except by means of a series of drawings.

In following the movements of melanophores, the neighboring epidermal cells were observed at the same time. It was found that the

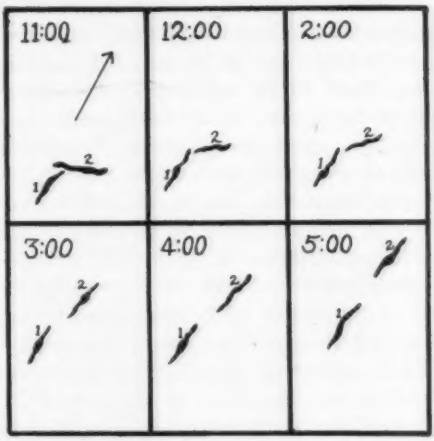

FIG. 7. First section of series of camera lucida drawings showing the paths taken by epidermal melanophores as they are carried toward a wound, coming to lie with long axes nearly perpendicular to the edge of the wound. The arrow indicates the direction of movement.

melanophores did not orient themselves irrespective of epidermal cells but moved with them and at approximately the same rate. Several investigators have concluded, from tissue culture studies, that melanophores possess the power of self-movement. The above described observations afford no ground for doubting that melanophores do have the power of self-movement, but there is every reason to believe that, under the conditions of the experiments in question, they are no more active than the other epidermal cells and that their radial arrangement depends upon the movement of the epidermal cells and not upon the activity of the melanophores themselves. Figures 7 and 8 are series of camera lucida drawings of groups of pigment cells which were near

healing wounds. The drawings show the successive positions of the melanophores as they became more nearly perpendicular to the edges of the wounds. The time intervals between successive drawings are indicated.

With regard to radial arrangement of pigment cells, there was a striking difference between auto- and homoiotransplants. There was never extensive radial arrangement around autotransplants. This is not strange, however, since in autotransplants there is little or no migration of epidermis after the epidermis of the host meets that of the graft. In fact, this is further evidence in support of the proposed explanation for radial arrangement.

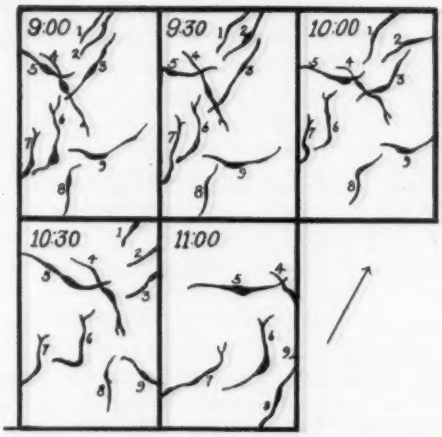

FIG. 8. Second section of a series of camera lucida drawings showing the paths taken by epidermal melanophores as they are carried toward a wound, coming to lie with long axes nearly perpendicular to the edge of the wound. The arrow indicates the direction of movement.

The writer wishes to express his sincere appreciation to Dr. H. W. Rand for his advice and aid throughout the period of this work.

SUMMARY

1. Immediately following the occurrence of wounds in the skin of tadpoles of *Rana clamitans*, the surrounding epidermis moves to cover the area. Wounds 5 mm. across may be covered in from 6 to 24 hours. The advancing epidermis is reduced to one or two cells in thickness, thus covering a larger area than previously. The epidermal cells become considerably elongated in the direction of movement. After the

advancing edges have met, the cells resume their previous shape. Subsequent mitosis restores the original thickness of the layer. There is considerable evidence for stereotropism in epidermal migration.

2. In wounds which cause a deficiency in the dermis, the epidermis thickens over the wound area until the smooth contour of the body-surface is restored.

3. In the region surrounding a healing wound there is temporarily a deficiency in the number of epidermal pigment cells due to their movement into the wound.

4. Autotransplants of white ventral skin transferred to the pigmented backs of tadpoles retained their specificity. They remained unpigmented for more than 100 days. Lateral and dorsal autotransplants in which the patches of skin were rotated through 90 or 180° also retained their specificity. There was no change in the pigmentation or the cellular structure of such grafts.

5. In all cases where homoiotransplants of white ventral skin were transferred to the pigmented backs of tadpoles the region of the graft became pigmented. Epidermal pigment appeared at the edge of the graft in an average of 9 days after the graft was made, and in the course of about 6 more days had extended to the center of the patch. Dermal pigment entered the graft area many days after the epidermal pigment had completely covered the patch.

6. The initial pigmentation of grafts results from a replacement of the graft epidermis by that of the host. The pigment cells are carried along by the host epidermis as it covers the patch. There is considerable evidence that the graft epidermis is destroyed by phagocytic action of the host epidermis. However, the epidermal layers of graft and host always unite immediately after the graft is made, indicating some initial affinity between the two layers.

7. Patches of skin which were grafted onto the ventral side of the body always degenerated. The ventral side of the body is perhaps an unfavorable place for grafts to receive nourishment.

8. The replacement of the epidermis of a graft by host epidermis is, essentially, a process of delayed wound healing.

9. In nearly all cases grafts, soon after transplantation, became reddened as the result of an enlargement of the dermal blood vessels of the graft. There is considerable evidence that the enlargement of the blood vessels is due to blood pressure which mechanically stretches the vessels in the absence of the normal tonus, the nerve connections having been cut at the time of transplantation. Later, and probably in consequence of restoration of nerve connections, the dermal vessels became reduced to normal proportions.

10. Radial arrangement of the epidermal melanophores occurs around homoiotransplants and skin wounds—that is, wherever there is translatory movement of the epidermis. In any migrating epidermis in which the melanophores are expanded, their long axes tend to become parallel to the direction of migration. The position of the individual melanophore, in radial or parallel alignment, is a consequence of the movement of the epidermal cells near a wound and is not due to independent orientation of the melanophores. The epidermal cells nearest the wound move first and most rapidly, therefore tending to rotate the elongated melanophores into the line of movement of the epidermis. Since the movement of the epidermis is centripetal, the melanophores become arranged radially around the point toward which the epidermis moves.

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OXYGEN DEFICIENCY AND SEWAGE PROTOZOA: WITH DESCRIPTIONS OF SOME NEW SPECIES[1]

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It is a common observation that the protozoa of hay infusions and other liquid media generally seek the top of the culture where oxygen is to be found. Investigation of a rich culture either in a jar or on a slide shows that only a few inhabit the oxygen-poor regions: *Metopus* among the ciliates; some of the very small flagellates and small amoebas, mostly of the limax type. Anaërobic protozoa are well known. Juday (1919) has described a freshwater anaërobic ciliate; Lauterborn (1908) has discussed a number of species, many of them bizarre forms, from the oxygen-poor waters of the Rhine. Lackey (1925, 1926) has listed twenty-nine species common in sewage containing little or no dissolved oxygen, but often abundant H_2S or CO_2; while Cole (1921) has discussed the oxygen supply of animals living under such conditions.

Most of the protozoa inhabiting sludge tanks are small and do not occur in great numbers—except those which live at the very top and are not considered in this paper, for they have an abundance of oxygen and may be transient forms in the tanks as well—so that they are not often noted by students unless a careful search is made. All of those ciliates characterized by Noland (1925) as living in water whose oxygen saturation is below 45 per cent have been encountered at one time or another, but usually in small numbers, with the exception of *Metopus sigmoides*. As a result of examining the protozoan fauna of the digestion tanks of five New Jersey towns, the Passaic River in some of its most polluted stretches, and septic tanks from three locations in Tennessee, it has been found that the same small group of protozoa is to be found in each of these situations.

Of the forms listed previously (Lackey, 1925) two ciliates and one flagellate were described as new species. The present paper includes four new flagellates and two new rhizopods. The wide distribution of this group argues that the factors limiting their occurrence are very largely those of anaërobism, for such conditions as pH, temperature, dissolved gases, and food substances must have varied widely in the

[1] Journal Series Paper N. J. Agricultural Experiment Station, New Brunswick, N. J. Dept. Sewage Research.

several locations examined. Sufficient check has been made on the first two of these conditions in sludge digestion to make this assumption plausible, and it is known that they certainly vary from time to time, and that the amounts of CO_2 and H_2S fluctuate considerably also in any given tank, apparently without a corresponding fluctuation of the protozoan fauna as a whole, in the same tank. It is also certain that while all sewage contains decomposition products which can possibly serve as food for the organisms, the variety of these substances is too great to be a probable limiting factor.

To ascertain the effects of dissolved oxygen on two protozoa characteristic of sewage disposal plants, tall cylinders were set up, so that compressed air could be forced through porous plates in their bases. This allowed aëration of the sewage (eventually producing activated sludge) or kept the sewage under completely anaërobic conditions. *Opercularia* sp., a large peritrichous holozoic ciliate and an active bacterial feeder, was selected as the obligatory aërobe, for it is abundant in the trickling filters, but never occurs in its active state in the tanks. *Trepomonas* was selected as the obligatory anaërobe, for it is characteristic of the depths of the tanks. Table I shows the behavior of these two forms in the cylinders under conditions varying from no aëration to constant aëration. Preliminary examination of the sewage at the treatment plant showed 3300 *Trepomonas* and 500 *Opercularia* per cc., and after standing in a refrigerator 24 hours there were 2900 *Trepomonas* per cc., and no active Opercularia. It will be noted that the sludge when examined at the disposal plant contained both active *Opercularia* and *Trepomonas*. There was only a trace of oxygen present. The sample was taken to the laboratory and put in the refrigerator and 24 hours later contained no dissolved oxygen. Its protozoan population had changed in this time; all colonies of *Opercularia* contained only closed-up individuals.

After 6 hours aëration 65 per cent of the *Opercularia* were active, whereas with no aëration none of the *Opercularia* were active. No count was made, but the normal population of *Trepomonas* seemed to be present. Under aërobic conditions, however, the 6 hours had reduced the numbers of *Trepomonas* almost 96 per cent. Many dead or apparently dead ones were seen. Their protoplasm was greatly vacuolated and the cells sometimes much above normal size.

Twenty-four hours of aëration was sufficient to cause them to disappear and they never reappeared in this sample. All the *Opercularia*, on the contrary, became active and showed an increase in numbers for 24 hours, then a gradual decrease. When the experiment was dis-

continued after 196 hours, there were few protozoa of any sort in either aërated or unaërated sewage, but most of the *Opercularia* were active in the aërated, although they presented a rather starved appearance, due probably to disappearance of their food.

Although the results practically speak for themselves, supporting evidence is easily obtained. During an examination of settled sludge from the Chatham, N. J., activated sludge plant, on August 2, the sampling showed no dissolved oxygen and there were present 9200

TABLE I

Numbers of Organisms Present With and Without Aëration
(Numbers refer to active organisms throughout)

TIME	AËRATED		UNAËRATED
		No. per cc.	No. per cc.
After 6 hours	Trepomonas	140	No count
	Opercularia	3200	0
After 24 hours	Trepomonas	0	3300
	Opercularia	12600	0
After 48 hours	Trepomonas	0	4500
	Opercularia	9300	0
After 120 hours	Trepomonas	0	5400
	Opercularia	8300	0
After 148 hours	Trepomonas	0	4000
	Opercularia	4500	0
After 172 hours	Trepomonas	0	2000
	Opercularia	4500	0

Trepomonas per cc., and only about 260 *Opercularia* per cc. The *Opercularia* were all closed up, inactive, while the *Trepomonas* were swimming about vigorously. This sample was then aërated for 3 hours, when an examination showed that all the *Opercularia* were actively feeding while about half the *Trepomonas* were killed and half active.

At the end of 6 hours aëration there were no signs of living *Trepomonas* in this cylinder, although the *Opercularia* were thriving and numerous small flagellates such as *Bodo, Cercobodo, Pleuromonas, Monas,* and *Tetramitus* were active. After 49 hours aëration a large

protozoan fauna was present, the small flagellates of the group listed above being 15,300 per cc., and *Trepomonas* being absent. In a control of unaërated sludge there were, per cc., 5900 *Trepomonas,* 900 *Tetramitus,* active, and perhaps 175 inactive *Opercularia.*

It is not intended to list the protozoa occurring in activated sludge or trickling filters or, in general, in sewage which contains much dissolved oxygen. But from the above experiments and similar ones, it is apparent that many protozoa encyst when swept into the oxygen-free water of a digestion tank or the bottom of a river heavily polluted with sewage; that like *Opercularia,* they are able to reduce their metabolic activities to a minimum and so survive for a time, at least, in this adverse environment. If conditions become favorable again, they excyst and become active. It is also seen that there are some forms which live under either condition and some which thrive only in the absence of oxygen. This latter factor is so sharply limiting that we find a small and cosmopolitan group of protozoa which may be regarded as common in Imhoff tanks, septic tanks, and the deeper waters of heavily polluted streams.

Table II does not include many forms which have been seen, but not sufficiently studied for proper identification. Some of these will undoubtedly prove to be new species when better known. It is therefore seen that careful investigation of this particular type of habitat will be productive of an acquaintance with a decidedly unusual and interesting group of protozoa.

Description of New Species

The following descriptions are concerned with two new rhizopods and four new flagellates, which have been found in studying the waters of sewage disposal plants and the water of a creek near Camden, N. J., which was heavily polluted with sewage. Only one of the flagellates, *Chroomonas cyaneus,* was found in the creek, the others being from sewage disposal plants. The new species are illustrated in Plate I, enlarged about 1000 times.

VAHLKAMPFIA FRAGILIS, Sp. Nov. Fig. 4.

Organism small, free-living, 5 to 15 microns long, naked, relatively abundant in some sewage, especially in cultures. Pseudopodia lobose, two to three in number, clear, steadily formed rather rapidly and always at end of cell opposite contractile vacuole and nucleus.

One contractile vacuole, always posterior to the nucleus, constantly present, emptying seldom. Nucleus always oval, flattened, with two to

three large endosomal granules (chromatin masses?) in center. Nuclear membrane delicate. Several hyaline spheres of varying size, oil or albumen, located in posterior region. Endoplasm finely granular, never extending into pseudopodia.

No flagellated stage found.

TABLE II

Group I

Present Only in Small Numbers or Infrequency in Digestion Tanks

RHIZOPODA

Dimastigamoeba gruberi
Vahlkampfia limax
Vahlkampfia guttula
Hartmanella hyalina
Vahlkampfia albida
Chlamydophrys stercorea

FLAGELLATA

Mastigella simplex
Cercobodo longicauda
Cercobodo crassicauda
Cercobodo ovatus
Monas amoebina
Monas minima
Monas vulgaris
Anthophysa vegetans
Helkesimastix faecicola
Bodo caudatus
Bodo lens
Bodo mutabilis
Pleuromonas jaculans
Oicomonas termo
Cyathomonas truncata
Dinomonas vorax
Tetramitus descissus
Tetramitus pyriformis
Hexamitus inflatus
Clautriavia parva
Euglena gracilis
Menoidium incurvuum
Peranema trichophorum
Distigma proteus
Petalomonas mediocanellata
Petalomonas carinata
Heteronema acus
Entosiphon sulcatum
Notosolenus orbicularis
Chilomonas paramecium

CILIATA

Hexotrichia caudatum
Colpoda inflata
Glaucoma scintillans
Plagiopyla nasuta
Colpoda cucullus
Cyclidium glaucoma
Paramecium putrinum

Group II

Always Present in Absence of Oxygen in Tanks

RHIZOPODA

Chlamydophrys minor, sp. nov.
Vahlkampfia fragilis, sp. nov.

FLAGELLATA

Mastigamoeba viridis
Mastigamoeba longifilum
Mastigamoeba reptans
Mastigamoeba radiosa, sp. nov.
Trepomonas agilis
Bodo glissans, sp. nov.

CILIATA

Holophrya sp.
Metopus sigmoides
Saprodinium putrinum
Trimyema compressa

Nutrition apparently saprozoic.

Division binary in active stage. Forms very small cysts, thick-walled, with a few slight protuberances. Excysts in fresh raw sewage (by putting cover-slips in Petri dishes of old cultures, cysts are collected which can be watched on being transferred to fresh material).

Rather common in five samples obtained.

MASTIGELLA RADIOSA, Sp. Nov. Fig. 2.

Animal with spherical body, about 20 microns in diameter, free-swimming or floating. Pseudopodia much like those of *Amoeba radiosa*, occasionally branching, clear, up to 80 microns long. Single flagellum up to 100 microns long, used with a lashing or whiplike motion. One contractile vacuole. Nucleus central, spherical with endosome, membrane very thin, chromatin granules very small. Cytoplasm finely granular, no zones being present. Several types of granules such as oil spheres, or crystalline bodies, present.

Some apparent binary fission stages seen. Nutrition saprozoic. Rather rare; found in two of the tanks examined.

MASTIGAMOEBA VOLUTANS, Sp. Nov. Fig. 1.

Body elongate, flattened, 25 to 30 microns long, with a flagellum, vibratile in anterior third, slightly tapering, one and a half to two times body length. Cortical layer of protoplasm clear, hyaline, endoplasmic portion finely granular, reticulate, with numerous small, square crystals and numerous spheres of varying size. Due to the fact that they stain with methylene blue and that in some of these animals a clear vacuole was found which contained from one to three of these spheres, they are assumed to be volutin.

Pseudopodia very short, numerous, rounded.

Contractile vacuole single, in posterior part of cell.

Nucleus near anterior tip of cell, round in outline, with a central endosome surrounded by minute chromatin granules within a clear zone of nuclear sap. Membrane thin, with a rhizoplast visible in the living animal, from the flagellar insertion to its surface.

Nutrition apparently saprozoic.

Reproduction not observed.

Somewhat common in samples from six localities.

CHLAMYDOPHRYS MINOR, Sp. Nov. Fig. 3.

A very small form, showing constant differences from *C. stercorea*. Diameter of the transparent shell is 20 microns. Cytoplasm finely

granular, extending out as a few finely-branching pseudopodia, which only occasionally anastomose and never become thread-like.

Contractile vacuole single, median. Nucleus centrally located, spherical, with a single endosome. A central zone of black, refringent, crescent-shaped granules is present.

Nutrition apparently saprozoic. Reproduction not observed. Movement slow.

Rather common in most sewage.

BODO GLISSANS, Sp. Nov. Fig. 6.

A somewhat flattened elongate organism, rarely over 20 microns long, cell of definite shape, but quite plastic. Two flagella, emerging on the ventral surface, anteriorly, from a lip-like fold. Swimming flagellum about body length, very tenuous and very active. Trailing flagellum slightly longer, thicker, used as an axis on which the animal glides. Forward movement rapid, path straight. When the animal changes its path, its amoeboid nature becomes evident.

No nucleus visible either in living specimens or those treated with neutral red or iodine. Some visible granules may be chromidia. One contractile vacuole, median. Protoplasm in anterior end mostly clear, but a few small spheres, probably oil, may be seen posteriorly. No visible kinetic apparatus. Reproduction by longitudinal division, while active. Nutrition saprophytic. No cysts observed. Rather common in sewage from several locations.

The animal is placed in the genus *Bodo,* following Calkins' (1926) key, but it is not holozoic, and the two flagella are not terminal but subterminal. None of the species described in Pascher's manual (1914) fit it. The position of the contractile vacuole and the inability to find an organized nucleus are also unique features.

CHROOMONAS CYANEUS, Sp. Nov. Fig. 5.

Organism pyriform, obliquely truncated anteriorly, tapering to a somewhat extended point posteriorly, never exceeding 10 microns in length. Two equal or subequal flagella emerge from a slight depression anteriorly. They are about body length, directed forward in swimming, and the path of the animal is a spiral. Two or more small contractile vacuoles are located at their base. There is no eyespot, nor can other structures be distinguished in this region. The nucleus is central and apparently there is no endosome, but only granular masses of chromatin. Chromatophores two in number, band-shaped, curved, of a bright blue color which speedily diffuses into the surrounding water when the animal

disintegrates. Pellicle thin, rigid. One case of binary fission while active was noted, but no cysts were found.

These organisms were not found in sewage, but were abundant in the waters of a creek, heavily polluted with sewage, near Collingswood, N. J.

Summary

There is an unusual group of protozoa to be found in the oxygen-poor and oxygen-deficient waters of sewage treatment plants.

Most of these are apparently facultative anaërobes. A few appear to be obligatory anaërobes.

It is shown that the presence of dissolved oxygen in sewage allows *Opercularia* to thrive, while the absence of oxygen is fatal to this protozoön, if the condition endures for several days. The reverse conditions are found to obtain for *Trepomonas*, except that active aëration of the water is quickly fatal.

Six new species of protozoa from sewage or polluted waters are described.

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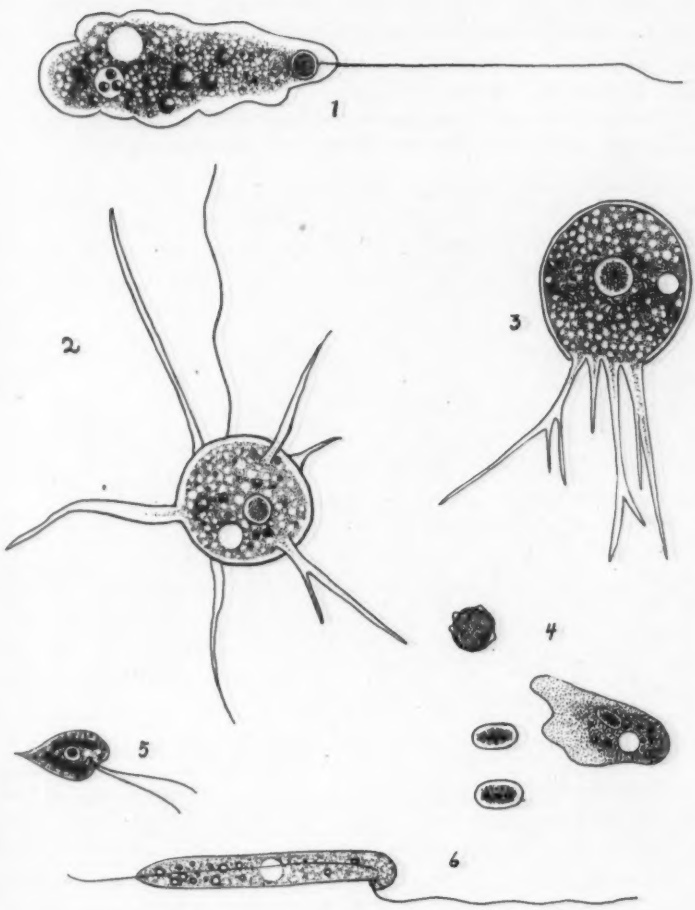

PLATE I

MODIFICATION OF TRAITS IN MOSAICS FROM BINUCLEATE EGGS OF HABROBRACON [1]

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Egg binuclearity has been suggested to account for the origin of a few rare mosaics in *Drosophila* and of hereditary mosaicism in *Lymantria*. Most of the mosaics which have been found in the parasitic wasp, *Habrobracon juglandis* (Ashmead), are likewise best explained by this hypothesis. The theory advanced by the writer (Whiting, P. W., 1922) is that after extrusion of the first polar body, the second oöcyte nucleus gives rise to two (reduced) nuclei which either take part in parthenogenetic cleavage (male mosaics) or segment after one has been fertilized (gynandromorphs). Post-reduction of binucleate eggs from heterozygous females would then result in mosaic males or, in case of fertilization of one nucleus, in gynandromorphs in which maternal contribution to male and female parts was different.

Mosaics in *Habrobracon* are very infrequent. The present paper is based on 132 mosaic males and 92 gynandromorphs which have occurred scattered through the cultures of various investigators. Whenever a mosaic has been reported it has been given a serial number in the "freak book" with a statement of its origin, a description, and any other pertinent data. The specimen, placed in a gelatine capsule, is then preserved in alcohol for future reference.

Mosaic Males from Heterozygous Mothers

Virgin females heterozygous for various genes have been bred in tests for linkage. Most of the mosaic males have been found in connection with these studies. They therefore arise from unfertilized eggs and are mosaic for one or more of those traits for which their mothers were heterozygous. It will be convenient first to give a record of these male mosaics as regards the various genes involved.

Females heterozygous for orange, *o* (eyes) (Chromosome I), have produced thirteen sons mosaic for this trait. When tested by mating

[1] The investigations here reported have been aided in part by grants from the Committee on Effects of Radiation on Living Organisms (National Research Council). The drawings for Plate I have been made by Kathryn A. Gilmore.

with orange females two of these mosaics had type (black) daughters, one had orange daughters, and one had both black and orange daughters. The line of division between black and orange ommatidia is difficult to determine, showing more or less gradation. In general the orange regions of eyes of mosaics appear darker than in normal orange and ocelli contain more or less brown pigment so that it is sometimes difficult to classify them as different from " wild type." Ocelli of normal " orange " contain more or less red pigment but not brown, while those of wild type contain much dark brown pigment.

Females heterozygous for ivory, o^i, have produced sons with mosaic eyes which may be classified as follows: nineteen had compound eyes described as mosaic for orange and black; nine for pale orange and black; five had one eye ivory, the other ivory grading through orange to black (Fig. 1); one had one eye black, the other ivory grading through orange to black. Observations were made on ocelli of thirty of these. They were classified as wild type, five; more or less brown pigment, three; very little brown pigment grading to colorless, five; wild type grading to much red pigment, three; more or less red pigment, ten; completely devoid of pigment, four. Breeding tests made of ten of these showed five breeding as black, three as ivory, and two as black and ivory. Twenty showed mosaicism for other traits for which the mothers were also heterozygous.

Females heterozygous for ivory have produced sons showing no very obvious mosaicism in eye color but which were mosaic in other traits of maternal origin affecting wings, legs, or body color. One with eyes and ocelli black bred as ivory; one with ivory eyes and ocelli bred as black and ivory. One with orange eyes and a trace of brown pigment in ocelli bred as ivory. One with orange eyes and ocelli bred as black. Six had pale orange eyes among which one had a trace of brown pigment in ocelli and bred as black; one had much red and brown pigment in ocelli; one had much red pigment in ocelli; while of the other three with colorless ocelli, one bred as black and one as ivory.

Females heterozygous for ivory produced aberrant sons showing no obvious mosaicism in any trait. Eleven of these had eyes classified as orange among which two had colorless ocelli while nine had much red pigment in ocelli. One of the former and six of the latter showed by breeding test that they were actually mutants to orange and one of the latter bred as black and was therefore a mosaic. Seven had eyes classified as pale orange among which one had no pigment in ocelli, two had much red pigment, while four had a small amount of red pigment. One of the last bred as black and was therefore mosaic and one bred as black stumpy and as ivory non-stumpy and therefore had mosaic gonads.

The sixty-two exceptional males produced by females heterozygous for ivory all showed orange modification of eye color to greater or less extent except that one which bred as ivory had black eyes; and one which bred as black and ivory had ivory eyes. The entire group includes forty-four from untreated stock and eighteen from mothers X-rayed as larvæ in twelve cases and as adults in six cases. Among the seven proved by breeding test to be mutants to orange only one was produced by an X-rayed (larva) mother. The seven mutants appeared entirely normal, showing mosaicism in no respect, not even as regards body pigmentation. There is no evidence that X-radiation has caused either modification or mutation to orange.

An orange-ivory compound virgin female, $o\,o^{i}$, produced a male (Fig. 2) with left eye orange, right orange dorsally, ivory ventrally with well-marked line of division between the two regions. Ocelli had red pigment granules characteristic of orange. The specimen bred as ivory.

Females heterozygous for miniature, *m* (body, antennæ, wings, legs) (Chromosome I), have produced two mosaic males showing clear-cut difference in antennæ, wings, legs, and general body size.

Females heterozygous for cantaloup, *c* (eye color) (Chromosome II), have produced fourteen mosaic males. Black and cantaloup regions are distinctly marked off in these mosaics in compound eyes and frequently even in ocelli in contrast to gradation observed between black and orange or black and ivory (Figs. 3–9).

Females heterozygous for long, *l* (antennæ, wings, legs) (Chromosome II), have produced eight mosaic males, one breeding as long. Difference was clear-cut, being evident in antennæ and wings, and could even be noted in legs.

Females heterozygous for narrow, *n* (wings) (Chromosome II) have produced three mosaic sons with clear-cut difference showing in wings.

Females heterozygous for Minnesota yellow, *My* (antennal segments) (Chromosome II), have produced five mosaic sons. The character is variable, modified by temperature, but the contrast is striking in antennæ of these mosaics.

Females heterozygous for reduced, *r* (wings) (Chromosome IV), have produced twelve mosaic sons. Difference is clear-cut and any one wing is either type or reduced. Tests showed four breeding as type and one as reduced. There were likewise produced from heterozygous mothers a reduced breeding as type and a type breeding as reduced, which were therefore also mosaic.

Females heterozygous for fused, *f* (tarsi, antennæ, wings) (Chromo-

some IV), have produced two mosaic sons. Another male mosaic for fused, found in an inbred culture (No. 3), was in every other respect similar to Stock 3, but proved sterile in observed matings with three females. The method of origin is uncertain, no other fused were found in the culture and it is therefore possible that this may have been a somatic mutant. The combination of traits is unmistakable and this specimen showed typical fused in tarsi, antennæ and wings. Besides this case the locus has been known to mutate four times. Modification of fused in mosaics will be discussed below.

A male mosaic for glass, *g* (eyes and antennæ) (Chromosome IV), occurred in a mixed culture so that parentage is uncertain. The left antenna was very thin as in typical glass, the right type. The eyes were each mosaic with clear-cut regional distinction between glass and type (Figs. 20, 21), but the outline of ommatidia near the margin departs from the normal hexagonal form probably due to absence of pressure from other ommatidia in development. The glass regions are genetically orange, the non-glass black. There is gradation of dark pigment into the orange regions as expected and outlines of bases of abortive ommatidia are rendered visible by the presence of this pigment.

A male mosaic for wavy, *wa* (wings), and a male mosaic for broad, *br* (thorax) (Fig. 16), have each been produced from a heterozygous mother (Chromosome V).

Two males mosaic for white, *wh* (eyes) and one for attenuated, *at* (antennæ) (Chromosome VI), have been produced by heterozygous females. White regions of compound eyes and of ocelli are, as in the case of cantaloup, sharply distinct from black, thus differing from genes in the orange series. (Figs. 10, 11, 12.)

Two mosaics, each with left eye strikingly banded, were produced by mothers heterozygous for ivory and for cantaloup. The normal brothers had eyes of the three expected colors,—black (*OC*), cantaloup (*Oc*), and white (ivory, o^iC, or ivory cantaloup, o^ic). Each mosaic had right eye orange or cantaloup in color which might be genetically cantaloup, *Oc*; ivory, o^iC, modified by the presence of *O* in the mosaic; or ivory cantaloup, o^ic. The last possibility is very unlikely since orange cantaloup, *oc*, is almost white and cantaloup with modified ivory, o^ic, should be no darker. The banded left eyes are shown in Figs. 13 (mosaic No. 550) and 14 (mosaic No. 540). Number 550 shows white dorsally bounded by a sharply marked-off narrow horizontal black band which is split anteriorly by an orange region into which it grades imperceptibly. A broad horizontal white band then follows, sharply marked off from the black lying dorsally, and likewise from a narrow horizontal black line below, which grades ventrally through orange to

ivory. Number 540 shows a somewhat similar pattern (Fig. 14), but the median light band is distinctly pink, "cantaloup." It seems most reasonable to suppose that the sharply marked-off light regions of these eyes are genetically cantaloup, *Oc*, whether they appear white or pink, since eyes of cantaloup stock vary from white to red, becoming progressively darker with age. The grading ivory or orange together with the black bounding bands are then ivory, $o^{i}C$, modified by the dominant allelomorph, *O*, in the cantaloup, *Oc*, regions. It is interesting to note that black color develops despite the absence of wild type, *OC*, tissue. Ocelli of No. 550 were colorless, which may have been either cantaloup or ivory, probably the former. In the case of No. 540, ocelli (Fig. 15) are mixed, the right ocellus as well as the right halves of the median and the left being colorless, "cantaloup." Brown pigment in the left halves of the median and left ocelli indicates modified ivory as in the left compound eye. Asymmetry in ocellar size and in pigmentation of ocellar region is to be ascribed to genetic difference in the tissues involved (Whiting, P. W., 1932).

Females heterozygous for stumpy, *st* (legs) (Chromosome VI), produced fourteen males mosaic for stumpy and three that were modified stumpy and thus suspected of being mosaics. Modifications of stumpy will be discussed below.

Males mosaic for various other traits the genes for which have not yet been shown to be linked have been produced by heterozygous mothers. These include two for semilong, *sl*, showing in antennæ, wings, legs; three for tapering, *ta*, showing in antennæ; one for yellow, *Y* (antennæ); twenty-one for shot vein, *sv* (wing veins); three for club, *cl* (tarsi); three for cut, *ct* (wings); one for indented, *in* (wings); one for attenuated, *at* (antennæ), one for twisted, *tw*, showing in antennæ. Certain males from heterozygous mothers have been suspected of being mosaic for other genes but traits are too fluctuating to assert this with certainty.

Origin of Traits in Gynandromorphs

Most of the gynandromorphs found in *Habrobracon* are from pure stock or among the progeny of recessive females crossed with dominant males. The reason for this is that they come only from fertilized eggs and that very few offspring are bred from mated heterozygous females or from dominant females by recessive males. By far the greatest number of mated females that have been set are recessive and are used in connection with studies of ratios of biparental males.

Thirty gynandromorphs have been found among progeny of orange-eyed defective, *d* (r_4 wing vein) females crossed with type males. While sex of antennæ can be readily determined, compound eyes vary

considerably in size so that there is no consistent sex difference. Color of eyes is, however, correlated with sex of antennæ. Among the fourteen with both antennæ male, eleven had orange eyes while three had eyes with some mixture of black and orange. Among the fifteen with one antenna male, the other female, eleven had eyes asymmetrical in color with the eye on the female side black, in which case the eye on the male side was orange or mixed; or with the eye on the female side mixed, in which case the eye on the male side was orange. Of the other four cases with asymmetrical antennæ, two had both eyes mixed while two had both eyes black. One wasp with both antennæ female had both eyes black. Male regions of eyes then are orange, matroclinous; female regions show dominant black, patroclinous trait, and are presumably biparental.

Ocelli are larger in male than in female and frequently in gynandromorphs there is asymmetry, the female side showing the smaller ocellus surrounded by integument with characteristically less pigment. Among twenty-seven of these gynandromorphs sixteen had large male ocelli, orange in color, while five had small female ocelli. Six had ocelli asymmetrical in size and color, larger (male) and lighter on one side. In three cases the male ocelli were typically orange while in the other three they had more or less brown pigment. In five cases the female ocelli were described as "black" while in the other one the color was "brown."

Figures 17, 18, and 19 illustrate distribution of pigment in mixed eyes of gynandromorphs from orange females crossed with black-eyed males. Number 373 (Fig. 17) had small (female) black ocelli, and female antennæ. It may be noted that the right eye is black (female) anteriorly. The left eye is orange (male). Number 375 (Fig. 19) is somewhat the reverse with male antennæ, large (male) orange ocelli, while the left eye is orange (male) anteriorly. The right eye is orange (male). Number 397 (Fig. 18) shows a banded condition in the left eye with orange male ocelli, male antennæ, and orange region anteriorly in the right eye. The grading margin may be noted between black and orange in these gynandromorphs in contrast to the clear-cut boundary between black and cantaloup or black and white.

Wings of males are smaller than wings of females. The gene for defective r_4 vein, *d*, permits fluctuation in the character. Grade 4 denotes the complete absence of the vein from a wing. Heterozygous females frequently show breaks classified as grades 1 or 2. Among the fourteen gynandromorphs with asymmetrical wings, the larger wings (female) had r_4 classified as follows: type 10, d1—3, d2—1, while the smaller wings (male) had r_4 classified: d2—2, d3—5, d4—7. In each

individual the male wing showed the greater defect indicating maternal origin.

We have already seen that mosaic males produced by mothers heterozygous for ivory usually show modification of this ivory color to orange. One gynandromorph (No. 322) from ivory female by black male had female abdomen and male head. Compound eyes were ivory showing no modification although female parts of body presumably were Oo^{i}. Ocelli were male with a trace of brown pigment. Another (No. 481) from a similar cross with head male, thorax and abdomen mixed, likewise had ivory eyes but in this case the ocelli (male) contained red pigment. Another (No. 605) from a female heterozygous for ivory crossed with a black male had male head, female abdomen. Eyes were pale orange, but ocelli (male) were colorless. Another (No. 304) from ivory female by black male had head mixed, abdomen female. Left antenna was female, right male. Eyes were pronounced orange dorsally, black ventrally. Ocelli were male and of light color but showing some brown pigment. Another (No. 296) from a female heterozygous for ivory by an orange male, had one antenna male, the other female. Eyes were ivory, ocelli male and colorless. Other gynandromorphs from crosses of females recessive for various traits by dominant males have shown male structures recessive (matroclinous) in the following cases; two for orange eyes, *o*; three for cantaloup eyes, *c*; three for long antennæ, *l*; one for type recessive to Minnesota yellow antennæ, *My*. Gynandromorphs from similar crosses have shown female structures, ocelli, dominant (patroclinous, presumably biparental), black in five cases of orange and in two cases of cantaloup.

Only four cases have thus far been reported indicating that maternal contribution to male and to female parts is different. These are cases of crosses of heterozygous females by recessive males. One involved reduced, *r* (wings), showing one reduced and one type primary. Three involving orange had black and orange regions in the eyes. Ocelli were orange and female in one (No. 513) so we may suppose the black parts of the eyes were male, the orange female. In the second (No. 602) the larger ocellus (male) was orange, the smaller two (female) were black. In the third (No. 526) the smaller (female) lateral ocellus had no pigment. It was in a darker (male) region. The median and left ocelli contained orange pigment and although large (male) were in a somewhat lighter (female) region.

Modification by Mosaicism of Fused and Stumpy Legs

The mosaic "mutant" to fused (No. 507) mentioned above had antennæ typical for fused. The left was slightly longer than the right.

Left wings were both type; right were both fused, the primary showing characteristic indentation at tip of radius vein, the secondary shorter than its mate on the left. The three left legs were type; the right showed tarsi with segments typically fused.

The propleuron was darker on the right side which was presumably composed of tissue bearing *f*. Since this specimen came from pure inbred stock it is likely that this pigmental difference is due to the factor *f* itself, in other words *f* is one of a number of genes causing darkening of integumental pigment. This mosaic proved sterile in observed matings with three females.

One of the mosaic males (No. 510) from a female heterozygous for fused had left antenna presumably type, but terminal segments were somewhat fused. Right antenna was fused and rather shorter than the average for this trait (Figs. 22 and 23). Primary wings were type but right secondary was short, probably fused. Left legs were type as was also the third right leg. First and second right legs were fused, the former showing much swelling in femur and tibia as is often the case in fused. Tarsus of this leg had joints completely fused (Fig. 24). Fusion of joints on the second right tarsus was incomplete (Fig. 25) so that it is possible to distinguish the five segments. The mosaic was highly fertile. Tests showed that it bred as wild type.

The other mosaic for fused (No. 490) was produced by a female which was also heterozygous for semilong, *sl* (antennæ, wings, and legs). The wings of this specimen indicate that the two types of tissue present were wild type and semilong fused, for the left wings were both normal while the right showed the combined influence of semilong and fused. Eyes and ocelli were all cantaloup, but this gene does not presumably affect legs or antennæ. Abdomen was normal male but external genitalia were missing (deficiency) except for a small clasper on the right.

Left antenna was type but showing fusion of joints terminally. Right antenna was fused but rather long, perhaps due to the presence of the gene *sl* (Figs. 28 and 29).

The prothoracic right tarsus was type, while all the others were fused but showing more or less segmentation (Figs. 30–34). The metathoracic right was essentially similar to the left. A metathoracic tarsus from stock fused is shown for comparison (Fig. 35).

It is evident that these three mosaics illustrate considerable modification in the fused tarsi. No such difference occurs in fused stock or in semilong fused. Presumably the change in the legs at least is due to mosaicism. Whether the modification of the type (?) antennæ is caused by mosaicism cannot be established from the two specimens.

Deficiency in genitalia of No. 490 may suggest correlated deficiency in antennæ (Whiting, P. W., 1926).

Distribution of type legs as associated with degree of modification of fused may be noted. In No. 507 there is no modification; all the type are on one side, all the fused on the other. In No. 510 all are type on the left. On the right the third leg is type, the middle tarsus much modified fused, while the first is unmodified fused. In No. 490 the five fused tarsi show modification but the middle right which is next to the type first leg is extremely modified in contrast to its mate on the left. The left mate of the type leg shows definite but relatively little modification. These relationships suggest that modification of fused may be greater according to proximity to type on the same side but that there is little if any influence across the median plane. It may be noted that in No. 490 in which there are no type legs on the left, the left antenna is type and the degree of modification is less extreme on this side. Little of significance may be based on these three specimens, but in view of the relationships in modification of stumpy, it is regarded as worth while to call attention to these facts.

In stumpy wasps the tarsal claws are very close to the tibia, the tarsal segments being reduced to minute chitinous vestiges (as in Fig. 39). Examination of over seventy-five specimens showed only six with a small tarsal segment on one or more legs. Legs showing this segment were prothoracic three, mesothoracic ten, and metathoracic one. There is therefore greater tendency for mesothoracic to possess a segment than for the other pairs. Moreover, an individual having a tarsal segment on one leg is likely to have it on others, for four of the six had segments on two legs while two had segments on three legs.

Females heterozygous for stumpy have been bred in X-radiation experiments by Raymond J. Greb. Among the progeny there have been produced fourteen males that were mosaic for stumpy and three which, although not possessing any type legs, showed modification of stumpy and were mosaic for other traits. Modified stumpy legs of mosaics are shown in Figs. 36, 37, and 38.

Of the entire group of seventeen, ten were produced by females X-rayed as adults, while seven were from non-rayed material. The modification of stumpy found in mosaics appears to have no relation to X-radiation of the mother.

Among the fourteen mosaics for stumpy there were thirty-six type legs and forty-eight stumpy. There were only two instances of right and left pairs of type legs but nine of the fourteen mosaics had all type legs on one side, all stumpy on the other. This distribution of type

and stumpy tissue is considered due to the distribution of genetically diverse nuclei in cleavage.

In order to have a quantitative measure of modification of stumpy, the legs of the seventeen mosaics (fourteen mosaic for stumpy) were roughly classified in grades from 0, denoting typical stumpy, to 6 with tarsi considerably over half normal length. The legs of the three mosaics that were not obviously mosaic for stumpy (with no type legs) were grade 0—10, grade 1—3, grade 2—4, grade 4—1.

Stumpy legs that were opposite to type legs were grade 0—23, grade 1—3, grade 2—3, grade 4—3. Stumpy legs that were on the same side as type legs were grade 0—2, grade 2—1, grade 4—7, grade 6—2. It therefore appears that modification of stumpy is due to association with type on the same side and that there is very little if any influence across the median plane.

If the grades of the various stumpy legs are totalled, the twenty prothoracic amount to 19; the twenty-four mesothoracic amount to 45; and the twenty-two metathoracic amount to 12. It therefore appears that mesothoracic are most subject to modification, prothoracic less, and metathoracic least. This order agrees with the six cases found in non-mosaics, but both ratio of legs modified and degree of modification is much higher in mosaics than in non-mosaics.

A Haplo-diploid Male Mosaic

A certain type of male mosaic not previously considered is a logical expectation from the fact that biparental (diploid) males appear in certain crosses and from the theory that a gynandromorph may be produced when one nucleus of a binucleate egg is fertilized.

Shortly after the discovery of the mutation orange, there were found five patroclinous males from orange females crossed with black males, which, although showing no obvious mosaicism, bred as orange and were highly fertile (Whiting, P. W., 1921). The hypothesis was at that time advanced that the "black" parts were haploid and strictly androgenetic. In view of the abundant evidence now accumulated for egg binuclearity and for male diploidism as well as lack of evidence for androgenesis, it seems preferable to regard these patroclinous male mosaics as haplo-diplonts, developing from binucleate eggs in which one nucleus was fertilized and gave rise, not to female parts as in a gynandromorph, but to diploid male parts.

Such a male, actually showing mosaicism, has recently been found and tested by Milton Franklin Stancati. An orange female (Stock 3) was crossed with a black-eyed male. This male was the original mutant

to indented, *in* (wings). In addition to the regular black females—eighteen, and the orange males—nine, there were produced seven black-eyed biparental males, two of which occurred in the same vial (d) with the mosaic (No. 544).

The mosaic was entirely male, with twenty-four segments in each antenna. Eyes were orange, matroclinous, except for black posterior region of the left. Ocelli were symmetrical in size and large (male). There was much brown pigment in left and median. The right had a very small amount of brown pigment (modified orange?). Body pigment was symmetrical.

The mosaic was highly fertile, producing seventy daughters when mated with three females which produced forty-three sons (female ratio 62 per cent). By the two orange mates there were produced fifty-four orange daughters, while by a black-eyed heterozygous sister there were produced ten black and six orange daughters including no indented although three indented males appeared and one non-indented, proving this sister heterozygous as expected. There were also no indented among numerous descendants of the mosaic by the Stock 3 females. The mosaic therefore bred like its maternal stock (No. 3).

Since eight of the twenty-six fertilized eggs developing in the fraternity of No. 544 produced males, a binucleate egg would here have a very good chance of producing a haplo-diploid male mosaic. In view of the fact that eye color alone has generally been used as the criterion of male biparentalism, it is highly probable that haplo-diploid male mosaics have been missed on account of failure of obvious mosaicism, being classified either as normal haplonts or as biparental males. On the theory advanced, their frequency may bear the same relation to gynandromorphs as the frequency of biparental males bears to females, —haplo-diploid male mosaics/gynandromorphs = biparental males/females.

A Case of Four-strand Crossing-over

Mosaic males from binucleate eggs would be expected to possess in their two types of tissue two of the various possible combinations of those genes for which their mothers were heterozygous. Only in those cases, however, in which the two or more pairs of genes affect the same structure can we be sure of what combinations are present. Thus, if eyes are mosaic for type (black) and cantaloup and wings are mosaic for type and long, there is no way of telling which wing is genetically cantaloup or which eye is genetically long. Virgin females heterozygous for long and for reduced have produced mosaic males having wings long on one side, reduced on the other, or having wings wild type on one side,

long reduced on the other. Gynandromorphs have likewise come from reciprocal crosses of orange and cantaloup having eyes type (black) if female, or showing the maternal color if male.

One instance of recombination of genes affecting the wings may be noted as it involves four-strand crossing-over in ovogenesis. In connection with her experiment on the production of impaternate females, Kathryn A. Gilmore bred progeny from a virgin female heterozygous for four factors in Chromosome II, *My/c.l.n.* Minnesota-yellow, *My,* an irregular dominant, affects basal segments of antennæ. It lies well to the left, 20 units ±, of the factor for cantaloup eyes, *c.* Long, *l,* lying 12 units ± to the right of cantaloup, shortens wings distally but lengthens antennal segments. Narrow, *n,* about three units to right of long, makes wings narrow. Among the progeny (male) segregating these four factors as expected there occurred a male (No. 552) with Minnesota-yellow long antennæ and cantaloup eyes and ocelli. Left primary wing was narrow (Fig. 26); right, long narrow (Fig. 27) with defective, r_4, venation. Character of antennæ indicates a crossover somewhere between *My* and *l.* Origin of defective is uncertain and need not concern us. Left primary wing indicates that a crossover occurred between *l* and *n.* The right primary, however, shows a non-crossover combination.

The facts indicate that we are concerned with a two-crossover oöcyte, two strands crossing over between *My* and *c,* the other two between *l* and *n.* There is no evidence for double crossing-over. Crossing-over between two strands in one region does not then prevent crossing-over between the other two in another region. For *My* and *n* we have pre-reduction; for *c* and *l* post-reduction. The two oötid nuclei involved in parthenogenetic cleavage were *My.c.l.n* and *My.C.L.n,* while *my.my.-Cc.Ll.Nn* went out in the first polar body.

Summary

Mosaic males from heterozygous mothers have shown clear-cut mosaicism for the recessive eye colors cantaloup and white. Orange, however, shows intergradation with black of wild type and ivory shows complicated types of modification and intergradation. A similar condition obtains in the case of gynandromorphs. Males have shown clear-cut mosaicism for sixteen other traits. As regards either fused or stumpy legs, however, there is much modification, with evidence that influence is from wild type tissue on the same side of the body, but not on the opposite side. Further evidence is presented indicating that gynandromorphs show maternal traits in male parts of body while female parts are biparental. Maternal contribution to male and female

parts may be different. A male mosaic has been found which is best explained as a haplo-diplont, being in part biparental. A mosaic male from a mother heterozygous for four linked genes indicates such a combination of traits that two crossovers must have taken place in the tetrad, one between two strands, one between the other two, in the egg from which this male developed.

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EXPLANATION OF PLATE I

Figs. 1, 2, 3, 5, 7, 9, 10, 11, 12, 13, 14, 17, 18, 19. ×27. In compound eyes solid black represents black, stippling indicates reddish or orange color, white unstippled regions are white.

Fig. 16. ×17.

Figs. 4, 6, 8, 15, 20, 21. ×64.

Figs. 22, 23, 24, 25, 28–39. ×27.

Figs. 26, 27. ×11.

Figs. 1–15 show eyes and ocelli of males mosaic for various traits as follows: Fig. 1. Type and ivory (No. 383). Fig. 2. Orange and ivory (No. 341). Figs. 3 and 4. Type and cantaloup (No. 378). Figs. 5 and 6. Type and cantaloup (No. 465). Figs. 7 and 8. Type and cantaloup (No. 351). Fig. 9. Type and cantaloup (No. 593). Figs. 10 and 11. Type and white (No. 601). Fig. 12. Type and white (No. 600). Fig. 13. Ivory and cantaloup (No. 550). Figs. 14 and 15. Ivory and cantaloup (No. 540).

Fig. 16. Mesonotum of male mosaic for broad, *br* (No. 465).

Figs. 17 (No. 373), 18 (No. 397), 19 (No. 375). Heads of gynandromorphs with male regions of eyes orange, female regions black.

Figs. 20, 21. Left and right eyes, respectively, of male mosaic for glass and for orange (No. 549). Stippling indicates brownish pigment. The glass regions are in general orange.

FIGS. 22 (left antenna), 23 (right antenna), 24 (right prothoracic leg), 25 (right mesothoracic tarsus) from male mosaic for fused (No. 510).

FIGS. 26 (left, narrow) and 27 (right, long narrow) wings of mosaic male (No. 552).

FIGS. 28 (left antenna), 29 (right antenna), 30 (left prothoracic tarsus), 31 (right prothoracic tarsus), 32 (left mesothoracic tarsus), 33 (right mesothoracic tarsus), 34 (left metathoracic tarsus) from male mosaic for fused (No. 490).

FIG. 35. Metathoracic tarsus of typical fused male.

FIG. 36. Left mesothoracic tarsus of male mosaic for stumpy (No. 495).

FIGS. 37, 38, 39. Two views of left mesothoracic tarsus and one of right metathoracic tarsus of male mosaic for stumpy (No. 497).

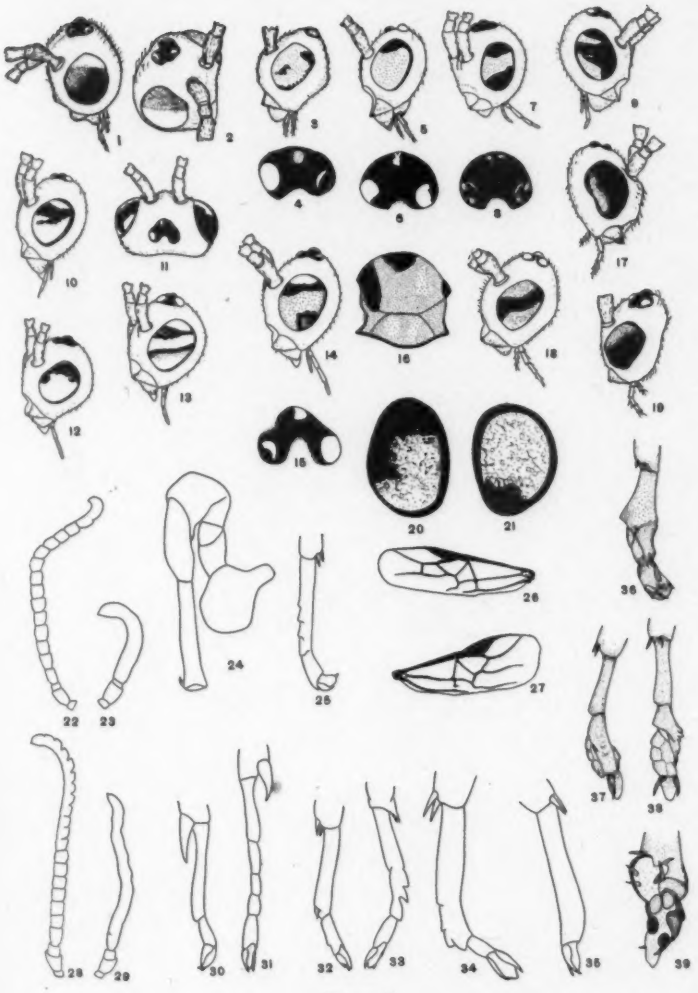

PLATE I

TEMPERATURE AND LIGHT AS FACTORS INFLUENCING THE RATE OF SWIMMING OF LARVÆ OF THE MUSSEL CRAB, PINNOTHERES MACULATUS SAY

JOHN H. WELSH

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Significant and numerous studies have been made in the past on the phototropism of plants and animals, and the quantitative aspects of the effect of light on photosensory systems have been extensively studied by Hecht and others; however, little is known concerning photokinesis and the effect of temperature on free-moving, light-sensitive organisms. Some investigators have even denied an effect of light on the behavior of such forms other than on orientation. Davenport and Cannon (1897) found an apparent difference in rate of swimming of *Daphnia* in full light as compared with swimming under one-fourth this intensity, but they concluded that this was probably due not to a change in velocity but to more rapid and accurate orientation at the higher intensity. Yerkes (1900) substantiated the findings of Davenport and Cannon on *Daphnia* and found in addition a slight effect of intensity on rate of swimming of *Cypris,* but he came to similar conclusions, namely, that the apparent change in rate was due primarily to changes in accuracy and rapidity of orientation. In neither of these investigations was a very wide range of intensities used. Their conclusions agree with those of the majority of observers before and since, with few exceptions. Moore and Cole (1921) found that the rate of locomotion of *Popillia japonica* during upward geotropic progression was influenced by light and that the rate of movement was a function of the light intensity. Cole (1922*a*) obtained similar results regarding the upward creeping of *Drosophila,* and (1922*b*) found a distinct effect of light on the rate of creeping of *Limulus.* Mast (1923) expresses doubt regarding the validity of Moore and Cole's (1921) results on *Popillia* for he states that they did not exclude the time required for the insects to orient and get under way. Mast also doubts the value of Loeb's (1890) observations on rate of movement of aphids as a function of light intensity, for he claims that temperature was not eliminated as a factor. Mast and Gover (1922) studied the effect of intensity of light and rate of locomotion of *Phacus* and *Euglena,* and although with

Euglena they found what may be a significant increase in rate at high intensities, they nevertheless concluded that light intensities sufficient to cause rapid and accurate orientation need not have any additional appreciable effect on rate.

The lack of agreement regarding the photokinetic effect of light, and the fact that the problem has considerable bearing on our proper understanding of movements of plankton organisms, led to an attempt to gain additional information. Crustacean larvae form one of the representative groups of the animal plankton of the sea and were found to be most satisfactory for the work as planned. The work was carried out during the summer of 1931 at the Woods Hole Oceanographic Institution. It was made possible largely through the kindness of Dr. H. B. Bigelow, Director of the Institution, and the author wishes to acknowledge his appreciation for the excellent facilities and equipment placed at his disposal. The author is also indebted to Professor W. J. Crozier for suggestions in the preparation of the paper.

Material and Methods

For the study of photokinesis a suitable animal should possess certain characteristics which have been lacking in part in many of the animals previously studied. They should be positive or negative to light; they should orient accurately and rapidly; they should move in a straight line; they should preferably be aquatic organisms, in order to make it possible easily to control the temperature. In addition to these requirements any satisfactory experimental animal must be obtainable in large numbers over a considerable period of time, or must be easily reared or kept in the laboratory.

For satisfying these requirements it is difficult to conceive of a more suitable form than the young larvæ of *Pinnotheres maculatus* Say, the mussel crab. The adults are found living as parasites in the mantle cavity of *Mytilus edulis,* the edible mussel. Sixty-five per cent of the mussels collected from a bed near Grassy Island, Woods Hole, were infested with these crabs so the adults were easily obtained. A considerable number of females carrying eggs were found at all times during July and August. These are easily kept in the laboratory in bowls of sea water, and one can have one or more batches of larvæ hatching daily, each batch containing several hundred individuals.

The larvæ are distinctly positive to light. They orient with head away from the light and by means of forward strokes of the swimming appendages move backward toward the light. This is similar to the orientation of the young larvæ of the lobster as observed by Hadley

(1908), and of the young larvæ of *Palaemonetes* as described by Lyon (1906). At all light intensities used there was extremely rapid orientation consuming only a fraction of a second, and the larvæ took a course toward the light which was a straight line in most cases.

At a given light intensity and temperature the velocity of movement was quite constant for individuals of a given age, but after two or three days the rate of swimming decreased and at the age of four to five days the larvæ had a tendency to be temporarily negative; this necessitated using larvæ of known age.

As a careful control of either temperature or light intensity is necessary when studying the effect of the other factor, all experiments were performed in a dark room; by means of a water-bath, the temperature could be accurately controlled, and with proper methods light of the desired intensity obtained.

The water-bath consisted of a 30-gallon insulated tank with 4 x 6 inch plate glass windows set in opposite sides and near one end. Temperatures below room temperature were obtained by means of a cooling unit similar to one described by Stier (1931), with a mercury thermoregulator operating a heating unit of 100-watt capacity. Rate as a function of light intensity was studied for the most part at temperatures slightly above room temperature which obviated the necessity of using the cooling device. The water in the tank was stirred by means of a motor-driven agitator, and was changed at frequent intervals in order to avoid loss of light by suspended particles which tended to accumulate in the tank.

The light-source was a 6-volt, 18-ampere, ribbon filament lamp, shielded by means of a double housing in order to prevent leakage of light. The light passed through lenses which kept the rays practically parallel. The intensity was controlled by means of Wratten neutral tint filters, which, used singly or in combination, transmitted the following percentages of the original light: 50, 25, 10, 5, 2.5, 1, 0.5, 0.1, 0.05 per cent. The beam of light passed through a series of screens with apertures of the proper size, and through ground glass for diffusion. A second light for attracting the larvæ to the opposite end of the trough consisted of a Spencer lamp with a 150-watt bulb and ground glass.

The larvæ were placed in filtered sea water in a trough of plate glass with inside dimensions 29 x 4 x 4 cm. This trough was covered with a glass plate and submerged to within a half centimeter of the top in the water of the bath. Here it was supported on hangers so that it was always at a given distance from the light source and so that the beam of light just covered the inside section of the trough and, the rays being parallel, reflection of light from the glass sides was negligible.

Measurements of the light intensity within the trough were made by means of a Macbeth Illuminometer, by putting a small test plate in the water of the trough. The intensity of light at the end of the trough nearest the ribbon filament lamp (the variable source) when no filters were used was found to be 93 meter candles. The 150-watt lamp at the distance used gave an intensity of 68 meter candles at the end nearest this lamp.

By means of the neutral filters it was possible, without changing the distance of the lamp from the tank, to obtain the following intensities of light at the end of the trough nearest the ribbon filament lamp: 46.5, 23.3, 9.3, 4.7, 2.3, 0.93, 0.47, 0.093, and 0.047 meter candles. The last intensity was the lowest that it was practical to use, for below this it was impossible to see the larvæ distinctly and even at this intensity it was necessary for the observer to be adapted to complete darkness before making each observation. There was, of course, considerable absorption of light by the sea water of the trough, and the intensity at the far end in each instance was considerably lower than the intensity given for the near end, and this varied throughout the trough; however, the total light reaching the larvæ as they swam from the far end to the near end of the trough was proportional to the intensity at the near end, and varied as this was varied.

In preliminary experiments observations were made on the rate of swimming of individual larvæ as compared with the rates of the first or middle member of a small swarm, and the variations were no greater in the second instance than in the first. As it was more often possible to complete a series of data if several individuals were used instead of one, a small swarm of 10–25 animals was used in most of the experiments. The writer realizes the possibility of greater variations in the results when swarms are studied but in this particular instance the behavior of several larvæ selected from a given batch showed as great uniformity as did single individuals, at least when the fastest and slowest members had been discarded.

In a particular experiment a group of larvæ were selected of the proper age and placed in the trough and adapted to the temperature of the water-bath for at least a half hour. They were then attracted by the 150-watt light to the end of the trough away from the variable light source, and their swimming movements would keep them in close contact with the glass in their endeavors to continue their course toward this light. This light would then be cut off and at the same time the light at the opposite end turned on and a stop watch started. The time necessary for the fastest individual or group of individuals to traverse the 29 cm. was then taken. At a given light intensity or temperature

either five or ten trials were made and the results averaged. In this way it was possible to determine the rate of swimming of the larvæ both as a function of temperature and light intensity. As stated above the extremely rapid orientation of the larvæ and the apparent absence of a latent period obviated the necessity of considering these factors and made the measurements easier and more accurate than would have been true with many free-swimming forms.

Temperature and Velocity of Larvæ at a Constant Light Intensity

Preliminary observations indicated a decided effect of temperature on the rate of swimming of the larvæ, and although only one series of measurements on individuals of the same age was made over a wide

Table I

Effect of temperature on rate of swimming of larvæ at a constant light intensity

Temperature	Time for swimming 29 cm. (Averages of 10 readings)	P.E. of time	Velocity $\frac{1}{\text{time for 29 cm.}}$
°C.	*seconds*		
27.0	12.38	± 0.100	0.0806
25.9	13.09	± 0.090	0.0763
24.8	14.53	± 0.046	0.0690
23.5	15.50	± 0.057	0.0645
22.0	17.70	± 0.099	0.0565
20.0	20.70	± 0.127	0.0483
18.5	24.79	± 0.175	0.0403
17.6	25.64	± 0.117	0.0391
16.5	27.72	± 0.139	0.0361
15.2	32.30	± 0.152	0.0310
14.2	36.11	± 0.149	0.0277
13.4	39.05	± 0.302	0.0256

range of temperatures, the results from this were quite significant. The range of temperatures used was from 27.0° C. to 13.4° C. The light toward which the larvæ swam was kept at a constant intensity of 93 meter candles. The results are shown in Table I. The times given for swimming 29 cm. are, at each temperature, averages for ten readings. Between different temperatures one hour was allowed for adaptation to the new temperature.

From these results one can determine the time for swimming a meter at different temperatures. At 27.0° this was 43.4 seconds, under the conditions of the experiment; at 18.5° the same larvæ required 1 minute 27 seconds for travelling a meter; and at 13.4°, 2 minutes 16.5 seconds.

Temperature changes in the sea are slow and comparatively small yet this factor must be of some importance in determining the rate of movement of plankton organisms, particularly when near the surface.

The probable errors of the times are shown in Table I and it may be seen that these are not large. There is a rather definite relationship between the average time for swimming 29 cm. and its probable error, and it is interesting to note that they are both affected by temperature in much the same way; as the mean time increases, the P.E. increases in proportion. The significance of this has been pointed out by Crozier (1929) and Navez (1930).

A plot of velocity against temperature centigrade (Fig. 1) indicates that the increase in velocity with increasing temperature is not on a simple smooth curve; it is evident that one curve does not fit the results. There is a break near 18.5° and near this point occurs the one velocity measurement which does not conform fairly well with the rest. It will

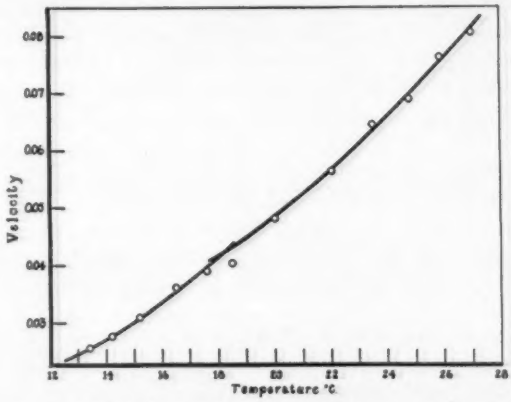

FIG. 1. Data of Table I plotted as velocity (reciprocal of time for 29 cm.) against temperature centigrade. Two curves are shown which intersect between 18° and 19°. This break is more evident when the same data is plotted as in Fig. 2.

be seen in Table I that the mean time from which this velocity was obtained has a high P.E. compared with those above and below. When the logarithm of the velocity is plotted against the reciprocal of the absolute temperature (Fig. 2) it is more apparent that between 13.4° and 27.0° two lines must be drawn to fit the data; these lines are straight and intersect at about 18.5°, thus indicating that the Arrhenius equation $\left[\frac{K_2}{K_1} = \epsilon^{\frac{\mu}{2}\left(\frac{1}{T}-\frac{1}{T_1}\right)}\right.$ where K_1 is the velocity at $T_1°$ Abs., and K_2 the velocity at $T_2°$ Abs.; $\epsilon = 2.718$; μ is a constant over a certain tempera-

ture range and designated by Crozier as a " temperature characteristic "] holds for the rate of swimming of these larvæ as a function of temperature. The values of μ as calculated are 16,900 below 18.5° and 12,800 above 18.5°. It is not necessary to go into the significance of these values and of the break, or critical temperature, for this has been done by Crozier (1924) and others, for many cases which are fundamentally similar; it is sufficient to note that corresponding values for μ have been frequently encountered in studies of the influence of temperature on rate of many biological processes.

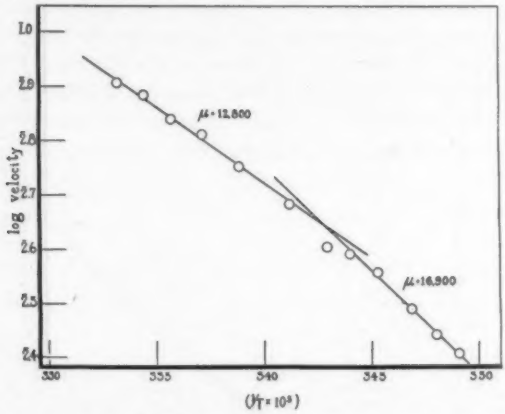

FIG. 2. Same data as in Fig. 1, plotted as the logarithm of velocity against the reciprocal of absolute temperature $\times 10^5$. That two lines must be drawn to fit the data is evident.

Few studies of the effect of temperature on the rate of locomotion of organisms have been made, the only other on a free-swimming form with which the writer is familiar is that of Glaser (1924) for *Paramecium*. He found that the Arrhenius equation could be applied, and secured a value for μ of 16,000 below 16° and of 8,000 above 16°.

It is realized that changes in density and viscosity of the sea water occur with changes in temperature and these factors enter in, and introduce slight errors in the measurements which are difficult to eliminate. To what extent they are significant remains to be seen.

The effect of temperature on the velocity of swimming at different light intensities will be discussed in a later section.

LIGHT INTENSITY AND THE RATE OF SWIMMING OF LARVÆ AT A CONSTANT TEMPERATURE

The importance of temperature as a factor influencing the rate of swimming of *Pinnotheres* larvæ has been indicated in the previous sec-

tion. During the work on the photokinetic effect of light the temperature was carefully controlled and kept constant, for a given series, within $\pm 0.1°$.

In addition to temperature, age was found to be an important factor influencing the rate of locomotion, and although this was not carefully investigated the age was taken into consideration in the later determinations of the effect of intensity of light. Because either age or temperature varied between separate series of experiments in some of the earlier work, it was impossible to average or compare much of the data, although the results were fundamentally similar. As an example of the effect of age on rate of movement it is necessary to cite only one instance. On August 23 at 2:00 P.M. a certain larva swam the 29 cm. in 14.2 seconds, in a light intensity of 93 meter candles; on August 24 at 10:00 A.M. the same individual required 15.2 seconds to swim the same distance. In each case the times as given are averages for five readings. It was also found that over a range of intensities, larvæ 30 hours old were less sensitive to the light than larvæ 15 hours old. It is obvious that age would not be of such importance if one were dealing with adult animals, but in the case of crustacean larvæ, as is well known, a few days makes a great difference in the responses of the animals to light (Hadley, 1908, and others).

Early in the course of the work on light intensity it was found that changes in rate of swimming were obtained over a comparatively small range of intensities. The maximum velocity, at temperatures near 23°, was reached at approximately 25 meter candles. This suggests that in much of the previous work on the photokinetic effect of light, intensities above the minimum necessary for eliciting a maximum response may have been used, and the conclusions that light intensity has no effect on rate of locomotion are perhaps unjustified in many instances. It is logical to assume that aquatic organisms are sensitive to a lower range of intensities of light than are land organisms, for they live constantly at reduced intensities.

It should also be pointed out that the intensity of light to which the animals are previously adapted affects to a certain extent the rate of movement in subsequent intensities. Several trials were made to determine the effect of dark adaptation on rate of movement in the light, with the expectation that for a few trials the animals would swim more rapidly, due perhaps to an accumulation of a photosensitive material in the light receptors. In every instance the first few trips, after dark adaptation of several hours, occupied more time than subsequent trips. This phenomenon is perhaps similar to that noted by Davenport and Cannon (1897), who found that *Daphnia* responded more quickly and accurately

to the light after having made several trips in it. Hecht (1925) also found in *Ciona* that after dark adaptation of several hours the first reaction time to a given intensity of illumination was definitely longer than those which followed, and which remained constant for long periods. These authors offered no explanation and as yet there seems to be no satisfactory reason why this should be true. The following experiment seems somewhat contradictory in view of these results on dark adaptation. Larvæ were adapted to a series of light intensities ranging from 0.093 to 93.0 meter candles and the time for swimming toward a light of 68 meter candles subsequently obtained. The results are shown in Table II. After adaptation to light of 0.093 meter candles 13.4 seconds were consumed in swimming 29 cm., in an illumination of 68 meter candles. This time increased as the intensity of the adapting

TABLE II

Effect of intensity of adapting light on time for swimming 29 cm. toward light of constant intensity. Age of larvæ 30 hours. Temperature 25.4° C.

Intensity of adapting light	Intensity of attracting light	Time for swimming 29 cm. toward light of 68 meter candles
meter candles	*meter candles*	*seconds*
0.093	68	13.4
0.93	68	13.3
4.7	68	13.9
9.3	68	14.2
23.3	68	14.2
46.5	68	14.3
93.0	68	14.7

light increased, until after adaptation to 93.0 meter candles 14.7 seconds were required for swimming the same distance. This indicates a distinct effect of the adapting illumination, and in the experiments to follow the larvæ were, in every case, adapted to light of a constant intensity before each trial.

From several series of experiments to determine the velocity of swimming in intensities ranging from 0.093 or 0.47 meter candles to 93.0 meter candles very uniform results were obtained. The maximum rate of swimming was reached in light of about 25 meter candles, and although this rate varied somewhat the variation was due to differences in age of the larvæ, temperature of the water, or in some instances perhaps to slight differences between given lots of larvæ or individual larvæ.

Table III gives one such series of data on larvæ 20 hours old, at a temperature of 24.5°. In this series readings were begun at the higher intensities and the intensities of the variable light source decreased by definite amounts between sets of readings. Progression from low to high intensities yields essentially the same results. In this particular series the faster individuals in the swarm travelled the 29 cm. in 16.6 seconds, at an intensity of 93.0 meter candles. At 46.5 meter candles there was no significant change. At 23.3 meter candles the time for swimming 29 cm. had increased to 17.5 seconds and from this intensity down to 0.47 meter candles, the lowest intensity tried, there was a gradual increase in time; at the lowest intensity the time being almost exactly twice as great as at the higher intensities.

A plot of this data as reciprocal of the time against the light intensity

TABLE III

Effect of light intensity on rate of swimming of larvæ at a constant temperature (24.5° ± 0.1). Series 8.6. Age of larvæ 20 hours.

Intensity	Time for 29 cm. (Averages of 5 readings)	P.E. of time	Velocity $\frac{1}{\text{time for 29 cm.}}$
meter candles	*seconds*		
0.47	33.9	± 0.91	0.0295
0.93	31.8	± 0.80	0.0315
2.3	27.2	± 0.70	0.0368
4.7	23.5	± 0.57	0.0426
9.3	21.5	± 0.62	0.0465
23.3	17.5	± 0.31	0.0571
46.5	16.7	± 0.41	0.0599
93.0	16.6	± 0.22	0.0602

yields a smooth curve as seen in Fig. 3. One point does not fall well on the curve but, as may be seen in Table III, the probable error of the time at this intensity (9.3 meter candles) is high in comparison with those above and below. The velocity at 93.0 meter candles is not shown on the graph as it is practically the same as that at 46.5 meter candles. It should be noted that in no instance were rates of swimming obtained in very low intensities or in total absence of light, for obvious reasons. It should also be pointed out that swimming movements of crustacean larvæ do not stop even in absence of light; instead the larvæ remain at or near the surface, kept there by constant but random movements.

One might expect, if the effect of light on rate of locomotion could be determined in the same way as the effect on orientation of the larvæ,

to find them obeying the Bunsen-Roscoe Law as did Loeb and Northrop (1917) in their investigation of the orientation of *Balanus* larvæ to a two-point source of light. If this law held for velocity of locomotion, the relationship between velocity and light intensity would be a linear one. That this is not true is quite evident, and the reason is perhaps obvious. Loeb and Northrop were concerned with the degree of turning of the path toward the stronger of two lights and this in no way depended on previous velocity and to only a very slight extent on water resistance.

The results obtained (Fig. 3) more nearly resemble those obtained

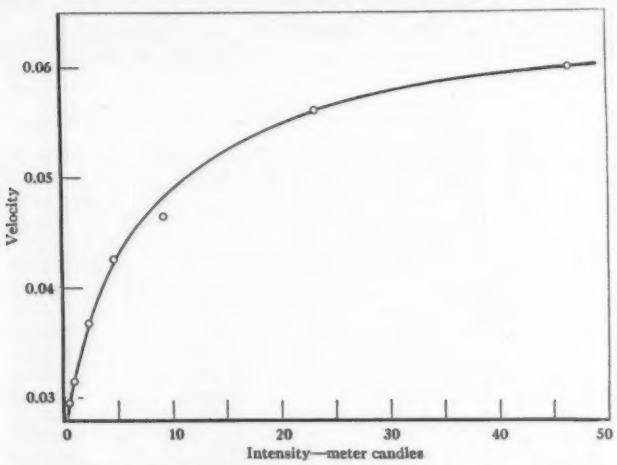

FIG. 3. Data of Table III, Series 8.6, plotted as velocity against intensity (meter candles). See text.

by Moore and Cole (1921) for rate of movement of *Popillia japonica* as related to light intensity, and later by Cole (1922) for *Drosophila*. In both of these instances they found apparent conformity with the Weber-Fechner Rule, as Henri (1912) had claimed for the reactions of *Cyclops* to ultra-violet light, and Patten (1915) when using a graded series of absolute intensities of opposed lights in studying orientation of blowfly larvæ. Although such conclusions are to a certain extent unwarranted by the fact that the Weber-Fechner Rule does not hold for intensity discrimination in certain forms, as pointed out by Hecht (1924, 1928), yet the approximate linear relation of response plotted against the logarithm of intensity is sometimes useful in analyzing such data. Crozier (1928) in discussing the case of *Limax*, where the

amount of turning per unit length of path is directly proportional to the logarithm of the light intensity, emphasizes the fallacy of considering this an obeyance to Weber's Rule and yet suggests that the same empirical treatment is useful, where other perhaps more significant treatments are impractical.

If the data given in Table III are plotted as velocity against the logarithm of the intensity, the relationship is found to be far from linear. This indicates that the velocity is not related directly to the logarithm of the intensity of illumination. In the work of Moore and Cole on the Japanese beetle they were dealing with an organism which, under a ruby light or in the dark, seldom showed any movement, but which was aroused to activity by illumination from any direction. As has been pointed out above, *Pinnotheres* larvæ are constantly moving even in total absence of light, and this initial velocity must bear some relation to later velocities produced by illumination.

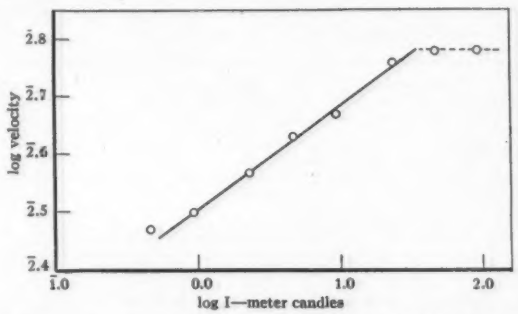

FIG. 4. Complete data of Series 8.6 plotted as logarithm of velocity against logarithm of intensity. The graph is essentially rectilinear until the maximum velocity of swimming is approached.

If we assume that the velocity of movement (V) is so related to the light intensity (I) that any increase in velocity (ΔV), produced by a small increase in intensity (ΔI), is a function of the velocity and also inversely proportional to the intensity, we obtain the following expression:

$$\frac{\Delta V}{\Delta I} \propto \frac{V}{I}.$$

Upon integration this yields the following equation:

$$\log V = k \log I - C$$

where k is the constant for the slope of the line, at any given temperature, and C is an integration constant. This expression indicates the way in

which the slope of the curve of velocity plotted against intensity depends, at any point, on both the previous velocity and light intensity. It may be checked by altering another variable such as temperature as is shown in the following section. If we apply this formulation to the data in Table III and plot the logarithm of velocity against the logarithm of intensity, we obtain a straight line over most of the range of intensities as shown in Fig. 4. Two velocities are shown at intensities of 46.5 and 93.0 meter candles which were obtained at or near the maximum velocity possible at this temperature, and which, of course, do not fall on the curve as drawn, but between 0.47 and 23.3 meter candles the straight line fit is good.

The theoretical significance of such an empirical treatment need not concern us. It is sufficient that we have a convenient method for comparing data obtained by varying the several factors such as temperature, light intensity, and age, which so evidently influence the rate of swimming.

TABLE IV

Effect of light intensity on rate of swimming of larvæ at different temperatures. Age of larvæ 15 hours.

INTENSITY	SERIES 8.21 A TEMP. 13.4° ± 0.1°		SERIES 8.21 B TEMP. 18.0° ± 0.1°		SERIES 8.21 C TEMP. 27.1° ± 0.1°	
	Time for 29 cm.	P.E. of time	Time for 29 cm.	P.E. of time	Time for 29 cm.	P.E. of time
meter candles	*seconds*		*seconds*		*seconds*	
0.093					22.8	± 0.28
0.47			39.2	± 0.62	18.3	± 0.28
0.93	86.7	± 2.16	35.9	± 1.04	17.1	± 0.06
2.3	64.7	± 1.06	31.0	± 0.39	15.2	± 0.21
4.7	56.8	± 0.89	27.0	± 0.35	13.7	± 0.09
9.3	47.7	± 0.53	23.9	± 0.32	13.5	± 0.11
23.3	39.7	± 0.30	22.4	± 0.33	13.3	± 0.17

THE EFFECT OF LIGHT INTENSITY ON RATE OF SWIMMING OF LARVÆ AT DIFFERENT TEMPERATURES

In the preceding sections we have shown the effect of temperature on the velocity of *Pinnotheres* larvæ at a constant light intensity, and the effect of light intensity at a constant temperature. Now it might be of value to compare light intensity curves from larvæ of the same age obtained at different temperatures.

Table IV gives three series of data on larvæ 15 hours old, at tem-

peratures of 13.4°, 18.0° and 27.1° C. The complete range of light intensities available was not used at each temperature, for the rate of swimming was extremely slow and rather irregular below an intensity of 0.93 meter candles, at the lowest of the three temperatures; however, sufficient determinations were made for adequate comparisons.

The curves for velocity plotted against light intensity are shown in Fig. 5. It may be seen that with increasing temperature, there is distinct displacement of the curves upward, and an earlier arrival at the maximum rate of swimming as the temperature increases. Also the slope of the curves changes with the temperature. At the lowest temperature the curve is much flatter than at the higher temperatures. While it was impossible to determine the effect of temperature upon

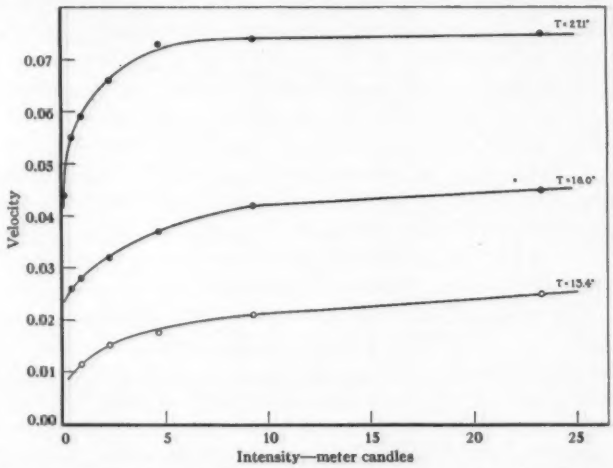

FIG. 5. Plot of data of Table IV as velocity against intensity. Open circles are Series 8.21 *A*; half-closed circles, Series 8.21 *B*; closed circles, Series 8.21 *C*. Temperatures as indicated.

the velocity of swimming in absence of light, it will be seen that there is a distinct effect on rate of locomotion in the dark, and if the curves were begun at the zero point on the abscissa, they would intercept the ordinate at varying levels above zero, depending upon the temperature. This is due to changes in rate of general activity, and to changes in the viscosity of the sea water. While no attempt has been made to correct for viscosity changes due to changes in temperature, these are of considerable importance. It was shown by Ostwald (1903, *a* and *b*) that in comparison with water, which might be considered to have a viscosity

of 100 at 0° C., sea water of 30 per cent salinity has a viscosity of 102, and this is reduced to 52 at 25° C. Thus sea water at 25° C. is approximately half as viscous as that at 0° C., and the same body would sink twice as fast at 25° C. as at 0° C. Viscosity changes with changes in temperature would therefore account for an appreciable part of the change in speed of swimming.

If we plot the data of Table IV as the logarithm of the velocity against the logarithm of the light intensity, we obtain as before essentially rectilinear graphs (Fig. 6), which vary in slope and in position relative to the abscissa. As in Series 8.6 (Fig. 4), the points representing velocities at or near the maximum do not fall on the lines, but over a definite range of intensities the straight line fit is good. The relative displacement of the graphs, and therefore the value of C in the

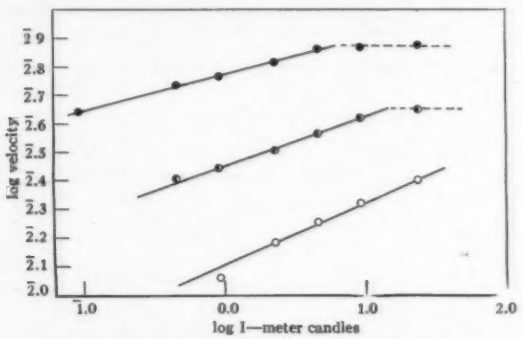

FIG. 6. Data of Table IV plotted as logarithm of velocity against logarithm of intensity. Symbols representing Series as in Fig. 5. See text.

expression $\log V = k \log I - C$, is seen to change considerably with a change in temperature. In addition, the slope of the lines is also seen to change. At 13.4°, k has a relative value of 0.225; at 18.0° $k = 0.175$; at 27.1° $k = 0.125$. At a given temperature the effect of increasing the intensity of illumination, within certain limits, is to increase the velocity. The changes in slope of the lines in Fig. 6 indicate the effect of temperature on the relation between velocity and intensity. At 27.1° the slope is less than at the lower temperatures, and the maximum rate of swimming is attained at a lower light intensity than at 18.0°. The expression

$$\frac{\Delta V}{\Delta I} \propto \frac{V}{I}$$

implies that if V be increased by operation of a variable independent of

I, the effect of increasing I must be correspondingly less—which is the fact. We have seen that temperature and light play an important part in determining the rate of locomotion of a crustacean larva. Other factors such as age and changes in viscosity of the surrounding medium must also be taken into consideration.

Summary

1. Larvæ of *Pinnotheres maculatus* Say are shown to be satisfactory animals for the study of photokinesis. The velocity of swimming is found to be greatly influenced by temperature and light intensity. Age, although not carefully investigated at present, is also an important contributing factor in determining the rate of locomotion.

2. A series of measurements of the effect of temperature on the velocity of swimming, at a constant light intensity, showed the applicability of the Arrhenius equation, and yielded values of μ of 12,800 above 18.5° and 16,900 below 18.5° C.

3. The larvæ are found to be sensitive to only a small range of light intensities. At temperatures between 20–25° C., the maximum possible velocity of swimming is attained at intensities between 10 to 25 meter candles. When velocity is plotted against light intensity a smooth curve is obtained. The same data when treated empirically according to the equation $\log V = k \log I - C$, yields essentially rectilinear graphs which are more satisfactory for a comparison of such data.

4. When series of measurements are made to determine the effect of light at different constant temperatures it is found that, besides a marked effect on general activity, there is a change in the relationship of velocity to intensity; the slopes of the curves change, and the maximum possible velocity of swimming for each temperature is reached earlier at the higher temperatures.

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BRANCHIAL RESPONSES TO ADRENALINE AND TO PITRESSIN IN THE EEL

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INTRODUCTION

The gill perfusion method (Keys, 1931*a*) affords a convenient means of investigating the physiology of the branchial blood vessels in the fishes. In particular, it would seem to be desirable to study the effects of various hormones on the effective calibre of the branchial vessels and on the performance of the branchial chloride-secreting mechanism (Keys, 1931*b*).

We have investigated the effects of adrenaline and of pitressin on the gills of the eel, *Anguilla vulgaris*, by means of the ventral aorta-gill preparation as described in a recent paper (Bateman and Keys, 1932). Throughout each experiment the perfusion pressure (mean of systole and diastole of the pump) was maintained at a constant level by adjustment of the pump stroke and the reservoir level.

Rates of perfusion were measured from the rates of inflow in some of the experiments and from the rates of outflow from the dorsal aorta in the other experiments. In the latter cases the dorsal aorta was cannulated at the level of the anterior portion of the liver. Practically identical results were obtained from these two methods. Venous escape from the cardinal and coronary systems was prevented in all cases except where specifically mentioned.

The perfusion fluid used was the same as that given by Keys (1931*a*, p. 359), the concentrations being $\Delta =$ about 0.72° for eels from sea water and $\Delta =$ about 0.60° for eels from fresh water. The external medium was either Plymouth sea water or Cambridge tap water, depending upon the medium in which the eels had been kept prior to the experiment.

The net chloride exchange between internal and external media in the gills was determined by analyses of ingoing and outgoing fluids, using Keys' (1931*c*) method. Analyses were done in duplicate or triplicate.

The effects of adrenaline were determined by the addition of adrena-

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line chloride (Parke Davis) to the perfusion fluid immediately before use so as to give adrenaline concentrations between 1/300,000 and 1/1,000,000. Pitressin (Parke Davis), the pressor principle from the posterior lobe of the pituitary gland, was used in concentrations ranging

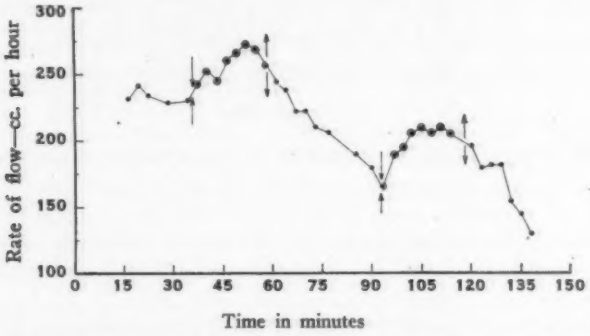

FIG. 1. The influence of adrenaline (1/1,000,000) on the rate of flow through the gills under constant pressure. The encircled points are from measurements during adrenaline perfusion, and the arrows indicate the introduction and removal of adrenaline.

from 25 to 50 international pressor units per liter by addition to the perfusion fluid just prior to use. The normal perfusion fluid was used as control in all cases.

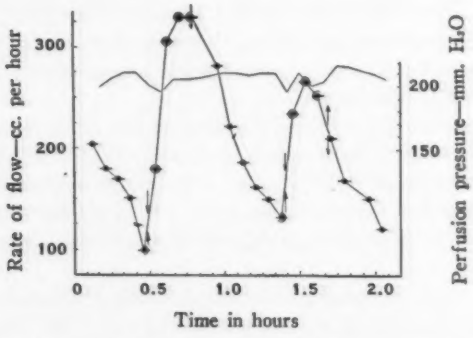

FIG. 2. The influence of adrenaline (1/500,000) on the rate of flow through the gills under constant pressure. The line drawn through the points shows the rate of flow. The encircled points are from measurements during adrenaline perfusion, and the arrows indicate the introduction and removal of adrenaline The continuous line shows perfusion pressure.

The Effect of Adrenaline on the Calibre of the Branchial Vessels

Adrenaline, in the concentrations used here, provoked unmistakable dilatation of the branchial vessels. Figures 1 and 2 show the pronounced increase in rate of flow through the vessels resulting from the addition of adrenaline to the perfusion fluid.

The experimental results shown in Fig. 1 and Fig. 2 are typical. The results of additional experiments are shown in Table I in which mean relative values for rates of flow with and without adrenaline are given. In each case the initial rate of flow without adrenaline is taken as 100 and all subsequent rates of flow expressed as percentage of the initial value.

TABLE I

Effect of adrenaline on the mean rate of flow through the perfused branchial vessels. All rates of flow expressed as percentage of the initial value.

Adrenaline concentration	Rate flow without	No. determinations	Rate flow with	No. determinations	Rate flow without	No. determinations	Rate flow with	No. determinations	Rate flow without	No. determinations	Rate flow with	No. determinations
1/300,000.....	100	3	143	10	128	11						
1/500,000.....	100	9	172	4	110	5	160	3	96	4		
1/500,000.....	100	6	116	10	91	9	96	6	75	7		
1/700,000.....	100	3	123	4	110	4	133	5	111	5	131	4
1/1,000,000....	100	5	112	7	103	4						

Adrenaline in these concentrations produces vasoconstriction in the vessels (other than the coronaries) of mammals. However, adrenaline in some concentrations (about 1/20,000,000) is known to have a small true vasodilator action (Dale and Richards, 1927) and it was thought necessary to test the effect on systemic vessels of the eel of the adrenaline concentrations used in the gill perfusions.

A preparation was made with the posterior part of the eel perfused from the dorsal aorta. The adrenaline effect was entirely similar to that obtained with the systemic vessels of the mammals, very pronounced vasoconstriction being obtained with adrenaline concentrations of 1/500,000 and 1/1,000,000. Figure 3 gives the results of an experiment of this type.

The final proof of the dissimilarity of the action of adrenaline on the branchial vessels and on those of the systematic circulation is provided by the following experiment. An eel is pithed and secured to the oper-

ating board. A mid-line incision is made exposing the heart and the ventral aorta. The sinus venosus is opened and the ventral aorta cannulated so that the course of the perfusion is through the gills to the systemic circulation and finally out through the opening in the sinus venosus. If, now, adrenaline is added to the perfusion fluid and the perfusion pressure is kept constant there is an almost immediate great reduction in the rate of flow, and removal of the adrenaline from the perfusion fluid restores the perfusion rate as soon as the adrenaline has been washed out of the system.

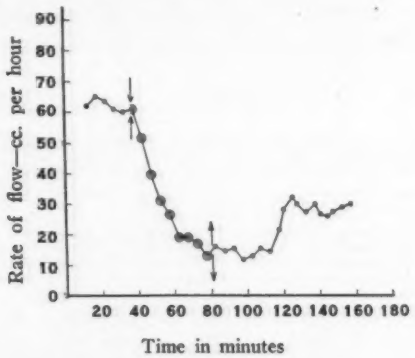

FIG. 3. The influence of adrenaline (1/500,000) on the rate of flow through the systemic vessels of the posterior part of the eel under a constant perfusion pressure of 35 cm. H_2O. The encircled points are from measurements during adrenaline perfusion and the arrows indicate the introduction and removal of adrenaline.

The effect of the adrenaline on the gills alone is demonstrated on the same preparation simply by cutting through the dorsal aorta at the level of the sinus venosus so that the perfusion is short-circuited past the systematic circulation. The results of this experiment are shown in Fig. 4.

THE EFFECT OF ADRENALINE ON THE CHLORIDE SECRETION

The apparent chloride secretion was always depressed by adrenaline in the experiments in which chloride concentrations were measured. The results of the two most complete experiments of this type are shown in Fig. 5 and Fig. 6.

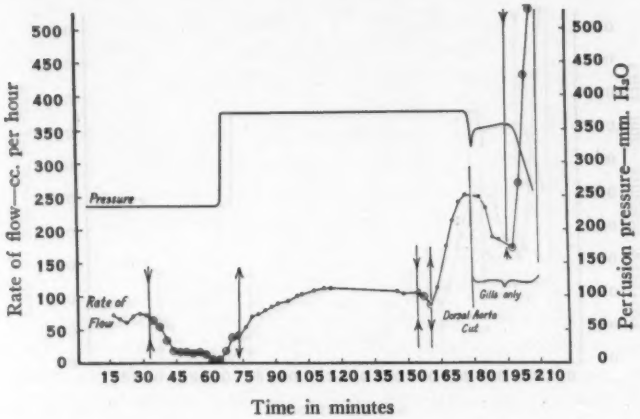

FIG. 4. A comparison of the adrenaline effect on the systemic and branchial vessels of the eel. The encircled points are from measurements of rates of flow during adrenaline perfusion and the arrows indicate the introduction and removal of adrenaline. See text for details of the experiment.

It will be noted that the adrenaline vasodilatation is very evident and closely parallels the very pronounced diminution in the chloride secretion. Figure 6 is particularly instructive; under the influence of adrenaline the secretion not only disappeared but the internal fluid became more concentrated during its passage through the gills. Table II, containing the condensed protocol of this experiment, will make clear the details of the method of calculation employed.

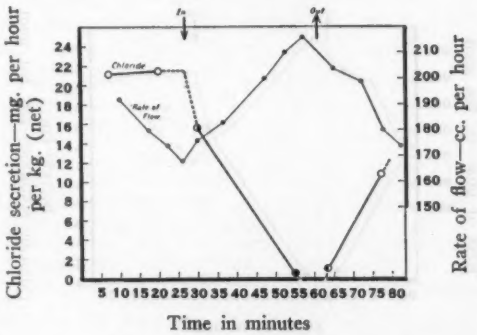

FIG. 5. The effect of adrenaline on chloride secretion in the gills bathed externally with sea water. Concentration of adrenaline, 1 in 1,000,000.

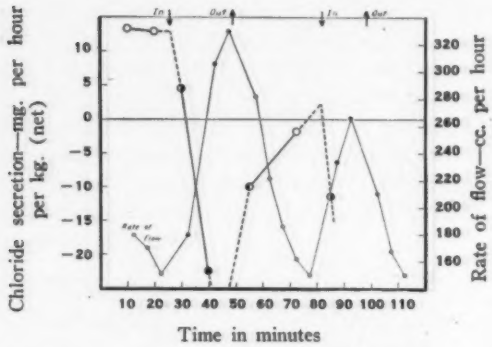

FIG. 6. The effect of adrenaline on chloride exchange in gills bathed externally with sea water. Concentration of adrenaline, 1 in 500,000.

The mean chloride change for all the experiments was 14.5 mg. in the periods with normal Ringer's solution and 1.9 mg. in the adrenaline

TABLE II

Protocol of Experiment 2A on the effect of adrenaline on the secretion of chloride by the perfused branchial vessels.

Eel from sea water, weight 370 grams. External medium, sea water; $\Delta = 2.0°$. Internal medium (1) normal; eel Ringer's solution; Δ = about 0.72°. (2) adrenaline; same as (1) but plus adrenaline chloride to give final adrenaline concentration of 1/500,000.

Internal medium	Time of collection of perfusate and control		Mean perfusion pressure	Mean rate flow	Chloride concentrations		Difference chloride	Net chloride change
	beginning	end			control	expt.		
			mm. Hg	*cc./hr.*	*mg./100 cc.*	*mg./100 cc.*	*mg./100 cc.*	*mg./hr./kg. eel*
Normal	1:05	1:15	20	202	693.0	691.4	−2.6	−13.4
Normal	1:15	1:25	21	160	693.0	690.0	−3.0	−13.0
Adren. (in gills about 1:31)	1:25	1:35	19	189	692.6	691.7	−0.9	− 4.6
Adrenaline	1:35	1:45	20	318	692.4	696.0	+2.6	+22.3
Normal (adr. out about 1:50)	1:45	2:05	20	278	693.8	695.1	+1.3	+ 9.8
Normal	2:05	2:20	20	166	693.5	693.7	+0.2	+ 0.9
Adrenaline (in gills about 2:25)	2:20	2:30	19	183	693.1	695.4	+2.3	+11.4
Adrenaline	2:30	2:40	19	259				
Normal (adr. out about 2:45)	2:40	2:50	21	189				
Normal	2:50	3:05	20	135				

periods, these figures representing the net loss of chloride from the internal medium per hour and per kilogram of eel. These values give really a minimum estimate of the difference between the apparent secretion in the two conditions owing to the fact that there was a considerable amount of overlap in many of the collections. In almost all cases the first period of collection of perfusate following a change in the perfusion medium represented, in reality, a mixture of perfusate from the adrenaline and the normal perfusion fluids.

The Effects of Pitressin on the Gills

The experiments with the pressor principle ("pitressin," Parke Davis) of the pituitary gland demonstrated only that this hormone, in the concentrations we used, has little effect either on the mean calibre of the branchial vessels or on the branchial chloride secretion.

In four experiments involving eight periods of normal perfusion fluid and six periods of pitressin (25 to 50 units per liter), the effect of the pitressin on the rate of flow was slight and variable. The maximum effect observed was a mean *decrease* of 17 per cent; the next greatest effect was an *increase* of 10 per cent. The average of all the measurements of rate of flow was very nearly the same with the normal perfusion fluid and with the pitressin. Taking the average of the non-pitressin periods as 100, the average for the pitressin periods was 97.

The chloride secretion by the gills appeared to be almost unaffected by pitressin and such effects as were observed were variable from one experiment to the next. In two experiments there was an increase in the chloride secretion during pitressin but in two other experiments the effect was in the opposite direction. In all cases the effect was small. The maximum change in the net chloride secretion during pitressin was an increase of 5.3 mg. Cl per hour and per kilogram of eel; the next greatest effect observed was a decrease of 4 milligrams.

Discussion

Wyman and Lutz (1932) have studied the effects of adrenaline on the blood pressure of the elasmobranch, *Squalus acanthias*. They recorded dorsal and ventral aorta blood pressures and found sustained pressor effects even with very small doses of adrenaline, which they interpreted ". . . as being due to extra-cardiac factors, peripheral to the gill capillaries, but the region of action of the adrenaline was not located." [8]

[8] Strictly, there are no true capillaries in the gills; see Keys and Willmer (1932) for the structure of the branchial vessels.

These results are obviously in complete agreement with our findings and we feel safe in predicting that suitable experimental technique would reveal adrenaline vasodilatation in the gills of the elasmobranch.

Since the completion of the present experiments we have discovered an interesting paper by Krawkow (1913) which apparently has escaped notice by other workers. Krawkow removed the gills of the pike and cannulated the stump of the ventral aorta. He estimated vasodilatation and constriction by perfusing and counting the number of drops from the cut ends of the gill bars, and he studied the effects of various substances with this technique. His results may be summarized in his own words (p. 603): "Von den untersuchten Substanzen bewirken Imidazolyläthylamin, Nikotin und Chlorbaryum Verengerung der Kiemengefässe. Coffein bewirkt nach kurzdauernder geringer Verengerung bedeutende Erweiterung der Gefässe. Chloroform erweitert die Kiemengefässe. Adrenalin, sogar in starken Verdünnungen, bewirkt sehr bedeutende Erweiterung die Kiemengefässe."

It would seem that the vasodilator effect of adrenaline in the gills of fishes is general. The similarity of this response to the adrenaline vasodilatation of the coronary vessels will have been noted. There is no longer any question as to the adrenaline vasodilatation of the coronary vessels (see Anrep, 1926, for literature), and Rössler and Pascual (1932) have shown that this is a direct response and is not due to an accumulation of metabolites. The latter possibility, of course, is eliminated in perfusion experiments like the present.

It is well known that the coronary arteries of the fish heart arise chiefly from the ventral ends of the posterior branchial vessels (Cuvier, 1805, and many subsequent workers,—see Grant and Regnier, 1926, p. 293) and, in fact, may be considered to be outgrowths of the branchial vascularization. Grant and Regnier (*op. cit.*) have assembled evidence to show that the coronaries in all probability are homologous throughout the vertebrates, ". . . the only important point of difference being the remote branchial origin in the lower vertebrates," (p. 294).

It does not seem to be unwarranted to suppose that the original specialization of vessels which respond to adrenaline by dilatation rather than constriction occurred in the gills of the early fishes. The gill most certainly began to evolve before the coronaries and it is difficult to see how an animal could survive if the vessels of its gills were subject to constriction at those critical moments of danger when adrenaline is liberated into the blood stream. If this view is correct, then the vasodilatory response to adrenaline characteristic of the coronary arteries of the higher vertebrates represents a useful heritage of the primitive condition in the gills.

There are two possible explanations of the effect of adrenaline on the chloride secretion. It may be, of course, that adrenaline simply paralyzes the secretory mechanism; the increase in the concentration of the internal medium during its passage through the gills which is observed in some cases following adrenaline (see Fig. 5) would then result from the normal permeability (Sumner, 1906; Smith, 1930; Keys, 1931*b*) of the gill membranes. This explanation is open to the objection that the indicated permeability may far surpass other estimates of the normal gill permeability (Keys, 1931*b*, p. 378 et seq.).

The alternative explanation for the effect of adrenaline on the chloride secretion is that the vasodilatation greatly increases the permeability so that the passive exchange may exceed the effect of the secretion. Such a relation between dilatation of minute vessels and permeability is apparent from the work of many investigators (see Krogh, 1929, p. 332) and would seem to be quite general. Krogh (*op. cit.*, p. 335) states: "I find no case in which a considerable dilatation has taken place without being accompanied by an increase in permeability."

We have compared the adrenaline response of the gill vessels to that of the coronary arteries; it may be instructive to make a similar comparison with regard to the effect of pitressin. Various workers have studied the effect of pituitary extracts on the calibre of the coronary vessels, but the results have not been consistent. Pal (1909), De Bonis and Suzanna (1909), and Dale (1909) reported vasoconstriction in mammalian coronaries, but Cow (1911) and Rabe (1912) could not confirm these results. Sumbal (1924) reported vasodilatation in the coronaries of the tortoise following administration of "infundin."

It seems that both the coronary vessels and the gill vessels may differ from the systemic vessels in not conforming to the general rule of vasoconstriction with pituitary extracts. It may be mentioned, however, that Drinker (1927) perfused the web of the frog with pituitrin (which is now known to be a mixture of the pressor and oxytocic principles of the pituitary gland) and found little effect on the diameter of the capillaries and no effect on the permeability.

In view of the pronounced effect of pitressin on the secretion of the kidney (Bugbee and Simond, 1928), it is of interest to note that no such effect on the branchial secretion was found in spite of certain other resemblances (Keys, 1931*b*, p. 382 et seq.) between the performance of the two organs.

Summary

Perfusion experiments are described in which the effects of adrenaline and of pitressin on the gills of eels were studied.

It is shown that adrenaline has a marked vasodilator effect on the gills whereas the same concentration of adrenaline has a powerful constrictor effect on the systemic vessels.

Adrenaline was found to decrease or abolish completely the branchial secretion of chloride; this effect is interpreted as being due to increased permeability of the gills associated with vasodilatation.

Pitressin did not appear to have any large or consistent effect either on chloride secretion or on the effective calibre of the branchial vessels.

The evolutionary and physiological relations between the gills and the coronary vessels are discussed. It is suggested that the adrenaline vasodilatation of the coronary arteries may represent a phylogenetic inheritance of a specialized vascular response originally developed in the gills.

Our best thanks are due to Professor Joseph Barcroft for his interest in these studies.

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